New insights into photodynamic therapy using porphyrin precursors

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New insights into photodynamic therapy using porphyrin precursors

Nieuwe inzichten in fotodynamische therapie gebruikmakend van porfyrine precursors

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1 General introduction

H.C. de Vijlder

NON- MELANOMA SKIN CANCER, SUNLIGHT AND RADICALS

Non- melanoma skin cancer (NMSC) is the most common cancer in Caucasians. The incidence of skin cancer continues to rise faster than that of any other cancer. More than 1 out of 6 Dutch inhabitants develops skin cancer (mainly NMSC) before his 85th year of life.[1] The majority of NMSCs are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). Non-melanoma skin pre-malignancies are Bowen's disease (SCC in situ) and actinic keratosis. In the last 25 years, the absolute numbers of patients in the Netherlands with first, histologically confirmed BCCs increased by about 7-fold in both men and women. In 2015, an incidence of more than 44,000 newly-diagnosed BCC cases is expected, increasing to more than 57,000 in 2020.[2] Subsequent BCCs in patients who have already been diagnosed with the first BCC are not included in these incidences, although the cumulative risk of developing one or more subsequent BCCs 5 years after diagnosis is 29%.[3] The burden of skin cancer is mainly disease burden, functional- and cosmetic problems and care consumption because of the good prognosis (quod vitam). General practitioners and medical specialists will be increasingly burdened with the inspection, the diagnosis and/or the treatment of suspect lesions in the future.[4]

Although several associations such as skin type, immune-compromised status e.g. organ recipients, viral infection and genetic background for skin cancer risk have been established, solar ultraviolet (UV) radiation is broadly accepted to be the main initiator and promoter of skin cancer. The spectrum of UV radiation may be divided into UVA (400-320 nm), UVB (320-290 nm) and UVC (290-200 nm). UVC is largely filtered out by the ozone layer and oxygen in the atmosphere. About 5% of the UV radiation that reaches the earth surface is UVB and about 95% is UVA. In particular, UVB has the potency to transfer its energy to (aromatic and heterocyclic) molecules promoting electrons to a higher energy level. Such excited molecules may activate oxygen leading to intermediate 'reactive oxygen species' causing damage to DNA, lipids and proteins. Neighbouring pyrimidine residues in DNA may form dimers. Such dimers that are characteristic in UV–induced skin cancers are often called mutations of the 'UV-signature type'.[5]

Reactive oxygen species (ROS) are obtained by the reduction of O_2 to $2H_2O$ requiring 4 electrons.[6] O_2 has a preference for univalent pathways of reduction, resulting in intermediate ROS (Figure 1). As the superoxide- and the hydroxyl radicals are very short lived they react rapidly with compounds in the direct environment. Initially, such radicals were thought to cause only molecular and cellular damage, mutagenesis, cancer and the degenerative process of biological aging. However, beside these detrimental effects it became clear that

living organisms adapted ROS to functional applications such as the involvement in signalling pathways that control gene transcription. In this way a delicate balance between the advantageous and detrimental effects of activated oxygen became an important aspect of life, and so ROS are controversial compounds, on the one hand causing damage, but on the other hand required for cellular metabolism.

Accumulation of genetic damage in relation to UV and ROS leads to increased mutations in p53 so that it will no longer restrain cell division, resulting in tumour growth. The p53 mutations are mainly of the 'UV-signature type' characteristic in UV–induced skin cancers.

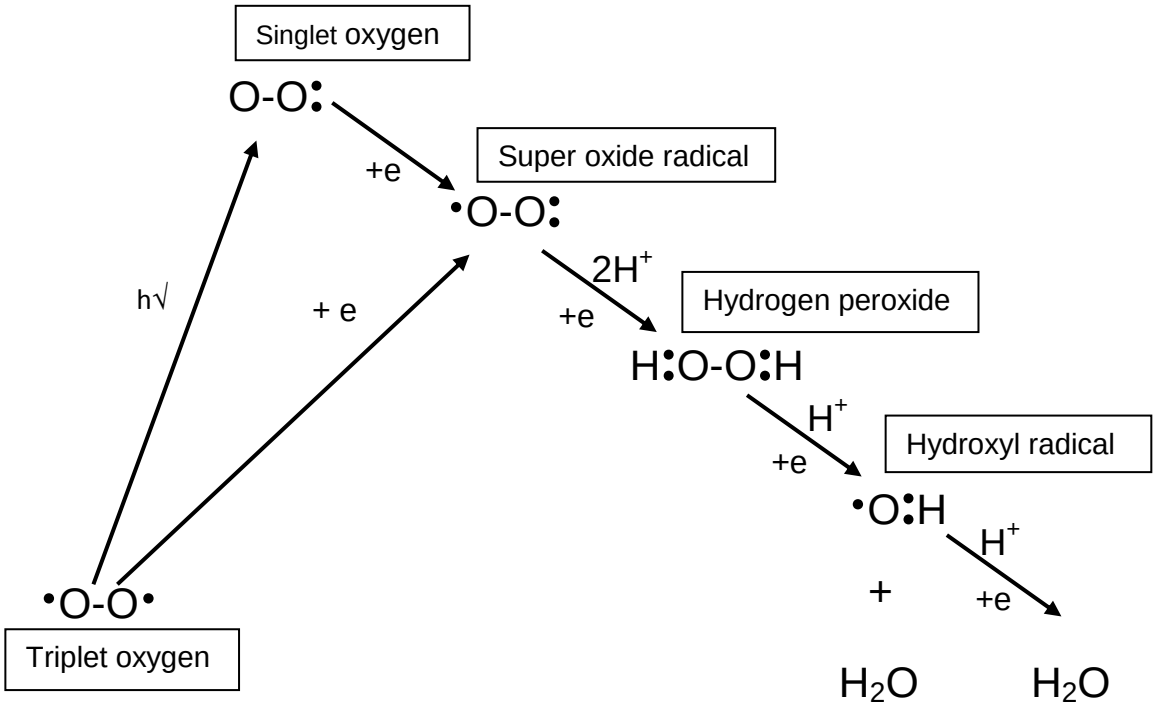

Figure 1: Pathways in the univalent reduction of oxygen to water leading to the generation of various intermediate reactive oxygen species (ROS).

NON-MELANOMA SKIN CANCER TREATMENT, PHOTODYNAMIC THERAPY AND REACTIVE OXYGEN SPECIES

In view of the increasing numbers of NMSC and the immense future work load for the physicians, treatment modalities that provide high cure rates and optimum functional and cosmetic results in the most cost-effective manner are imperative. Current treatment options include excision, curettage and electrodessication, cryosurgery, radiotherapy, topical chemotherapy (5-fluorouracil) and immune modulating agents (imiquimod), photodynamic

therapy (PDT) and Mohs' micrographic surgery. Conventional surgical excision is currently considered the gold standard.

The choice of treatment for BCC depends on the patient and the tumour characteristics. Important prognostic factors for recurrences are histological subtypes (morheaform and micronodular types), location in the H-zone, irradically-excised BCCs and BCC larger than 20 mm in diameter. The standard treatment for BCCs is surgical excision with a 3 or 5 mm margin, depending on the histological subtype and the diameter according to the Dutch dermatologic oncology guidelines.[7,8] Mohs' micrographic surgery is an efficient and tissue preserving treatment option for high-risk BCCs and recurrences. The key to Mohs' micrographic surgery is the excision and the control of the complete peripheral and deep resection margins in one plane allowing orientation, mapping and re-excision of microscopic tumour extensions. These extensions can be followed without sacrificing inappropriate amounts of normal tissue, yielding high cure rates and maximum preservation of tissue. Radiotherapy is an alternative treatment option for large primary BCCs at locations where preservation of anatomic structures is desirable. Both Mohs' micrographic surgery and radiotherapy are time-consuming as compared with the conventional surgical excision. Alternative non-invasive treatments for superficial BCCs are PDT and topical application of 5-fluorouracil or imiquimod. The topical treatments are less effective, but have several advantages such as better cosmetic outcome and more skin preservation over excision. Curettage, electrodessication and cryosurgery are treatment modalities with high recurrence rates and should only be used for small low-risk BCCs. A new treatment option for patients with locally advanced or metastatic BCCs that are not amenable to surgery or radiotherapy may be treatment with vismodegib (GDC-0449), a first-in-class, small-molecule inhibitor of the Hedgehog pathway. A phase 1 study of vismodegib reported a 58% response rate in patients with advanced BCCs.[9]

The Dutch dermatologic oncology guidelines for SCC recommend surgical excision with a wide margin (5-10 mm) as the first choice treatment.[10] Radiotherapy and Mohs' micrographic surgery are treatment options that can be used in specific circumstances (e.g. functional and cosmetic results). Treatment with topical 5-fluorouracil or imiquimod and PDT may be considered for Bowen's disease (BD) and actinic keratosis.

Photodynamic therapy (PDT) is a cancer treatment modality that is increasingly usedfor thin NMSCs (superficial BCCs, BD and actinic keratosis) and for (palliative) therapy of other malignancies such as head and neck-, Barrett's oesophagus-, prostate-, bladder- and gastrointestinal cancers.[11-17] Treatment consists of two relatively simple procedures. The administration of a photosensitive drug or a precursor and the illumination of the tumour with visible light. The cytotoxic effect of PDT is the result of the photochemical reaction that is

initiated by the absorption of light of an appropriate wavelength by the photosensitizer in the presence of oxygen. A simplified diagram (Figure 2) shows the possible pathways of the energy absorption and dissipation. Using conventional light sources, a single quantum (hv) of light is typically absorbed by the photosensitizer molecule, which is electronically excited transforming from its ground state (PS_0) into an excited singlet state (PS_1). PS_1 may re-emit radiation as fluorescence that can be used clinically for photo-detection. However, in order to obtain a therapeutic photodynamic effect PS_1 must undergo electron spin conversion to its triplet state PS_3. This triplet state is relatively long lived and hence exchanges the energy through collisions with molecules in its environment. Two specific types of reactions have been described. The type I reaction involves the collision with substrates or solvents producing reactive free radicals or radical ions, which subsequently can interact with oxygen to produce oxygenated products. Type II reaction involves the collisions with the ground state oxygen molecules ($^3O_2$) forming the highly reactive singlet oxygen ($^1O_2$) (Figures1 & 2).[17,18] Generation of reactive oxygen species (ROS) via type II is mechanically simpler than via type I and most photosensitizers are believed to operate via type II mechanism. Singlet oxygen is a highly reactive oxygen species with a short lifetime (~10-320 ns) limiting its diffusion to only approximately 10-55 nm in cells.[19] For these reasons the primary target of PDT is determined by the distribution of the photosensitizer in the cells and the tissues. The responses of tissue to PDT may be divided into three main categories of cellular-, vascular- and immunological responses. The mechanism of action of tissues to PDT resulting in the overall response is a combination of these three.

PHOTODYNAMIC THERAPY AND THE CELLULAR RESPONSE

Photodynamic therapy (PDT) may evoke three main cellular death pathways. These are apoptotic, necrotic and autophagy-associated cell death.[20] Apoptosis is generally a major cell death modality in cells responding to PDT. Apoptosis is a type of programmed cell death. It is morphologically characterized by chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, cell shrinkage, membrane blebbing and formation of apoptotic bodies without plasma breakdown. In the final stage of apoptosis phagocytes remove dying cells without eliciting an inflammatory response. At the biochemical level apoptosis entails the activation of caspases.

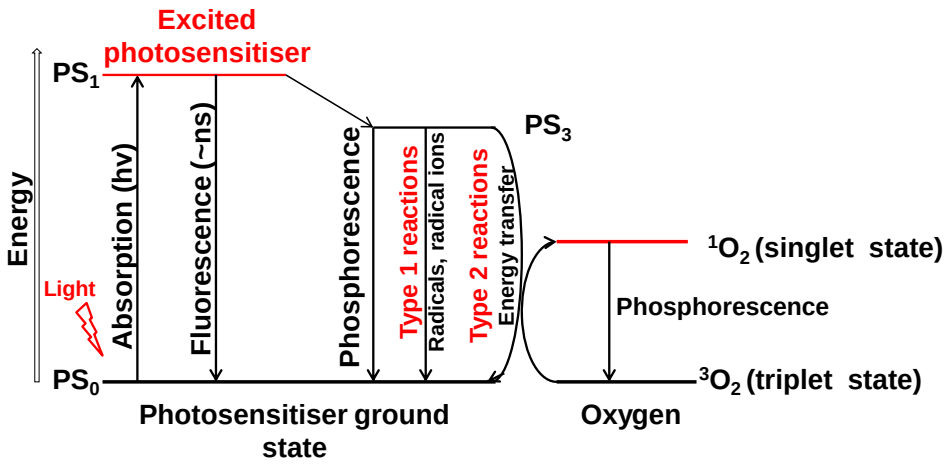

Figure 2: Jablonski diagram illustrating the electronic states of photosensitiser and oxygen as well as the transitions between them.

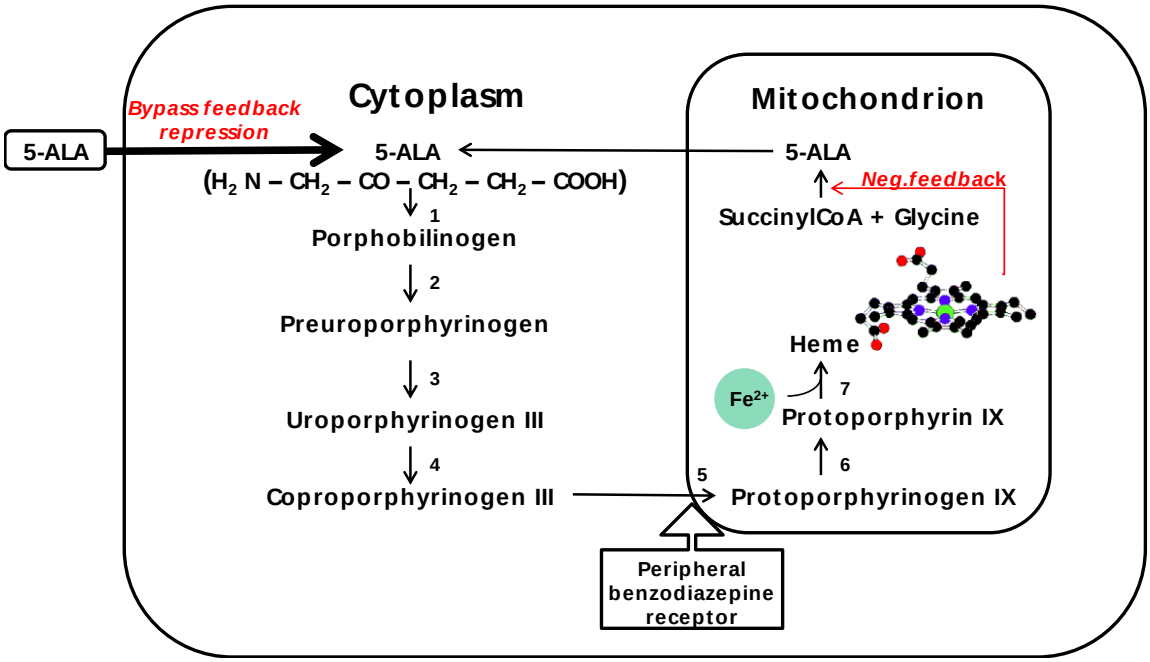

Figure 3: Schematic figure of intracellular protoporphyrin synthesis from ALA.
Enzymes in haem synthesis:
1: porphobilinogen synthase; 2: uroporphyrinogen synthase;
3: uroporphyrinogen III cosynthase; 4: uroporphyrinogen decarboxylase;
5: coproporphyrinogen oxidase; 6: protoporphyrinogen oxidase;
7: ferrochelatase.
Symbols: ● carbon; ● oxygen; ● nitrogen; ● ferrous ion

Necrosis is a disorderly cell death that is thought to be the result of pathological insult or may be caused by a bio-energetic catastrophe, ATP depletion to a level incompatible with cell survival. It is morphologically characterized by vacuolization of the cytoplasm, swelling and breakdown of the plasma membrane resulting in an inflammatory reaction due to the release of cellular contents and pro-inflammatory molecules. The biochemistry is characterized by the absence of caspase activation, cytochrome C release and DNA oligonucleosomal fragmentation. Autophagy is a process by which cells undergo partial auto-digestion through the lysosomes. It is characterized by a massive vacuolization of the cytoplasm. In adult organism, autophagy functions as a self-digestion pathway promoting cell survival in an adverse environment and as a quality control mechanism by removing damaged organelles, toxic metabolites or intracellular pathogens.

PHOTODYNAMIC THERAPY AND THE VASCULAR RESPONSE

Photodynamic therapy (PDT) induced damage has been shown to lead to different vascular responses such as vasoconstriction or dilatation, adhesion of thrombocytes and leucocytes and leakage of tissue fluid and macromolecules.[21] Changes in vessel diameter and platelet aggregation are responses that occur early during PDT. These responses may be reversible. Leakage from vessels causing oedema is usually observed immediately after PDT. The PDT induced collapse in the microvasculature may lead to severe hypoxia.[22] Tumour vessels are usually more sensitive to PDT- induced damage than normal vessels. It is suggested that the vascular response causing indirect damage by the deprivation of oxygen and nutrients (PDT-induced hypoxia) is necessary in addition to the direct cellular PDT damage to achieve complete tumour destruction.[23,24] The vascular effects of PDT may vary considerably depending on the photosensitizer and the drug-light interval that is used.[25]

PHOTODYNAMIC THERAPY AND THE IMMUNE RESPONSE

Photodynamic therapy (PDT) is known to interact with the innate and adaptive immune system. Preclinical and clinical studies have demonstrated that PDT eliminates tumours by direct tumour cell death and indirectly by augmenting anti-tumour immunity. PDT induces local- and systemic inflammation which is characterized by a rapid influx of neutrophils and an increased expression of pro-inflammatory cytokines (e.g. interleukin 6). The role of neutrophils seems to be critical in the response to photophrin-PDT; in neutrophil-depleted mice the response is markedly decreased, but in ALA-PDT influx of neutrophils is observed, however it is not critical in the response.[26,27] The long-term efficacy of PDT depends on the presence of an adaptive immune system.[28] Initial studies in mice models demonstrated that long term tumour control is diminished in immune-compromised mice. A decreased anti-

tumour immunity has also been observed in clinical studies.[29] Apparently, there is a role for the immune system, but the exact mechanism requires further investigation.

PRINCIPLES OF PHOTODYNAMIC THERAPY USING PORPHYRIN PRECURSORS

One approach to PDT is the use of porphyrin precursors such as 5-aminolaevulinic acid (ALA), which has been under investigation for over 2 decades.[30,31] ALA is converted within cells to the photosensitizer protoporphyrin IX (PpIX), via the haem cycle present in every cell with mitochondria (Figure 3). The chelation of iron to PpIX to form haem, is relatively slow. Exogenous administration of ALA bypasses the feedback suppression of haem on the ALA synthesis from succinyl-CoA and glycine. As a consequence, it overloads the haem cycle resulting in a temporary accumulation of PpIX. Although the haem pathway is present in all cells with mitochondria, some tissues accumulate more PpIX than others.[30] A high accumulation of PpIX is generally found in tissues derived from ecto- and endoderm like epidermis, oral mucosa, endothelial cells, endometrium, urothelium or glands. In contrast, tissues of mesodermal origin such as muscle, connective tissue, cartilage and blood cells show low PpIX accumulation. Two subcellular localisation studies reported that PpIX fluorescence was pre-dominantly observed in the mitochondria.[32,33] The uptake of ALA was accelerated in tumour cells as compared with that in normal cells.[34] Moreover, a higher PpIX accumulation was observed in malignant cells as compared to that in its normal counterpart cells.[35]

ALA-PDT has been used topically for the treatment of NMSC,[15] for cancer in the oral cavity[36] and gastrointestinal tract.[37] The advantage of topical ALA-PDT over other types of PDT using pre-formed photosensitizers is the absence of widespread skin photosensitization, except on the application area. Initially high (80-100%) complete response rates were reported for topical ALA-PDT in the treatment of superficial NMSC. [30,31,38-40] However, a considerable variation in the long-term response rates was observed. Substantially lower response rates were reported in a few small scale long-term response rate studies.[15] The variation observed in the efficacy of ALA-PDT especially in thick and large lesions could be ascribed to a number of factors that limited the response. The availability of ALA to the cells in the deeper areas of the skin lesions is thought to be limited by the penetration depth of topically applied ALA.[41] Attempts to enhance ALA tissue penetration and PpIX synthesis, in order to improve the cure rates of NMSC included pre-treatment curettage, pre-treatment with dimethylsulphoxide (DMSO), prolonged ALA application, intralesional ALA application, the use of EDTA, desferrioxamine, differentiation-modulating agents such as methotrexate and calcitriol to enhance PpIX formation.[42-44]

A different approach to improve the uptake of ALA is the synthesis of more lipophilic pro-drugs of ALA. ALA is a zwitter-ion at physiological pH, with a positive charge on the amino group and a negative charge on the carboxylic acid group (Figure 4). Such hydrophilic compounds are assumed to have limited capacity to cross biological barriers such as cell membranes and the stratum corneum. ALA esters, which are more lipophilic than ALA were introduced in order to achieve deeper penetration. The more lipophilic methyl ester of ALA, methyl-aminolaevulinate (MAL) is registered in Europe under the trade name Metvix®. Although more lipophilic, elevated intracellular PpIX concentrations after MAL administration in comparison with ALA could not been observed either *in vitro*[45] or *in vivo.*[46] No clinical trials comparing the responses between ALA-PDT and MAL-PDT have been reported to date. Increased intracellular PpIX concentrations were observed *in vitro* for longer chained ALA esters such as hexyl-aminolaevulinic acid (HAL). However, an *in vivo* study showed varied results on PpIX induction after HAL application.[47]

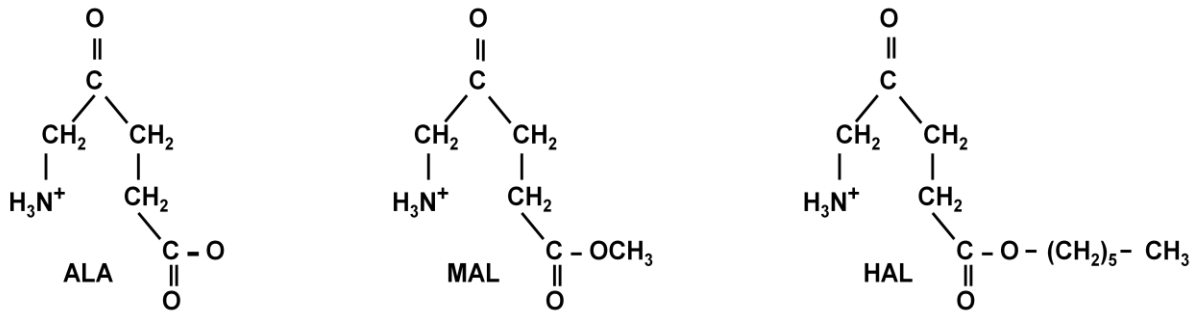

Figure 4: Molecular structure of ALA (5- or δ-aminolaevulinic acid = 4-oxo, 5-amino pentanoic acid) , MAL (methyl-aminolaevulinate) and HAL(hexyl-aminolaevulinate)

An important difference between ALA and the ester derivatives of ALA is their tissue distribution because of their different biophysical and biochemical characteristics. The cellular uptake of MAL is different from that of ALA. ALA is taken up via active transport using β-amino acid and GABA carriers, whereas ALA esters are presumably taken up by passive transport, [48] although active transport by other transporters than for ALA cannot be excluded. While the vehicle in which the precursors are dissolved may also effect the distribution, it seems less important since Moan et al.[49] used the same vehicle for both MAL and ALA and still showed a different distribution. PpIX fluorescence was detected at distant sites after topical ALA application, whereas it remained within the application site after MAL application. They concluded from this observation that ALA was systemically distributed after topical application, whereas MAL was not.

OPTIMIZATION BY LIGHT FRACTIONATION

The actual PDT response depends on three important factors. These are the concentration of PpIX in cells, the presence of oxygen and the delivered light dose. The plateau concentration of PpIX in cells is limited by the haem synthesis pathway and the photo-bleaching of PpIX limits the light dose that can be delivered during a single treatment session. That is why ALA-PDT using a single illumination is not optimal. In order to improve the efficacy, PDT treatment is often repeated after several days.[50,51] MAL-PDT using the Metvix® protocol is repeated one week after the first treatment. Another approach to increase the response of ALA-PDT is to modify the illumination scheme by splitting the illumination into two light fractions separated by a dark interval (fractionated PDT). The interest in fractionated ALA-PDT was awakened almost 2 decades ago by the clinical observation by W.M. Star that PpIX re-appeared in the time after the therapeutic illumination [W.M. Star, personal communication]. The original rationale behind the design of light fractionated ALA-PDT was the utilisation of the PpIX that was re-synthesised after the first illumination. This led to a series of pre-clinical studies investigating the response of tumour and normal tissues to fractionated ALA-PDT.[52-56] In different model systems, light fractionation proved to be effective as compared with single illumination. Moreover it was shown that the increase in response to light fractionated ALA-PDT was the highest when a low dose fraction was followed by a high dose fraction, separated by a dark interval of two hours. The choice of the fluence for the first fraction was critical and a high fluence for the second light fraction and a dark interval of more than 90 minutes were required for maximum tissue response. The results of pre-clinical studies were confirmed in randomized clinical trials.[57,58] It was observed that PpIX continued to be synthesised *in-vivo* after the first illumination[59-61] and that cells in tissues within the treatment volume including the surrounding tumour vasculature were responsible for this re-synthesis.[62] However, there was no correlation between the overall amount of PpIX that was re-synthesised and the effect of light fractionation.[52,63]

Other studies performed in order to elucidate the mechanism underlying light fractionation were guided by the results from two related *in-vivo* studies investigating the differences between the response following ALA and MAL. Fractionated MAL-PDT did not lead to an increased response *in vivo*. Moreover, light fractionated ALA-PDT led to the formation of acute oedema in the first few hours after treatment whereas light fractionated MAL- PDT did not.[64] These results and the above mentioned observation that ALA is systemically distributed after topical application whereas MAL is not[46] indicated that endothelial cells may be involved in the mechanism underlying the increased response. Historically the vascular

effects associated with topical ALA-PDT received relatively little attention, but are now recognised as playing a potentially important role.[65]

Recently, the increased response to fractionated ALA-PDT was reproduced *in vitro*.[66] Intriguingly, the efficacy of light fractionation strongly depended on the concentration of PpIX at the time of the first light fraction. Only cells incubated with low concentrations of ALA showed increased death rate in response to light fractionated ALA-PDT. It was suggested that the delivery of a small PDT dose followed by a dark interval led to PpIX re-localisation and/or conformational changes of molecular targets that may have increased the susceptibility of cells to undergo apoptosis or autophagy.[67-72] This increase in efficacy of light fractionation was not observed at high ALA concentration in the medium.[66] The delivery of high PDT doses may overwhelm these pathways and lead to cell necrosis. Given these insights it was hypothesized that cell populations such as endothelial cells in the deeper dermis exposed to lower concentrations of ALA may be more susceptible to light fractionation.

ADVANTAGES OF FRACTIONATED ALA-PDT IN THE DAILY PRACTICE

The high prevalence and the increasing incidence of non-melanoma skin cancers (NMSCs) impose an ever increasing financial burden on the healthcare system. Therefore, health economic evaluations assessing the treatments for NMSCs are becoming highly important. A study on the cost-effectiveness of MAL-PDT in the treatment of actinic keratosis and BCCs based on socio-economic and health economic models[73] indicated that MAL-PDT was a cost-effective intervention in the treatment of actinic keratosis as compared with cryotherapy, whereby the response was expressed as clinical response plus excellent cosmetic outcome. Moreover, MAL-PDT had better value for money than excision in the treatment of BCCs. In a recent comparative cost minimization study, MAL-PDT according to the Metvix® protocol, topical imiquimod (Aldara®) and 5-fluorouracil (Efudix®) were compared with each other in the treatment of superficial BCCs. Imiquimod was more effective than MAL-PDT and 5-fluorouracil. Cost-effectiveness evaluation showed that imiquimod and 5-fluorouracil were more cost effective than MAL-PDT. The cost minimization analysis showed that 5-fluorouracil was a cheaper treatment for superficial BCCs than imiquimod.[74] Although there are no studies available to date on the cost-effectiveness on fractionated ALA-PDT, we believe that fractionated ALA-PDT is more cost-effective than MAL-PDT, based on the lower costs per patient and the high efficacy.[57] ALA gel is less expensive than Metvix® (MAL): (67 € /gram magistral preparation, Fagron® versus 149 € /gram Metvix®), ALA gel needs to be applied only once instead of two times as that for Metvix® cream. Moreover, fractionated ALA-PDT requires only one day of treatment, instead of two days according to the Metvix® protocol.

AIMS OF THIS THESIS

This thesis is based on earlier publications[75-77] and the aims of the research described were to investigate:

1. The long-term efficacy of the fractionated ALA-PDT in the treatment of non-melanoma skin cancer.
2. The possible mechanism(s) responsible for the increased efficacy of fractionated-PDT by examining the differences between ALA and MAL.
3. The efficacy of MAL-PDT as compared with the surgical excision (gold standard therapy) in the treatment of non-melanoma skin cancer.
4. Genetics of human skin pigmentation variation and the risk of skin cancer.

REFERENCES

1. de Vries E, Nijsten T, Louwman MW, Coebergh JW. Skin cancer epidemic in the Netherlands. *Ned Tijdschr Geneeskd* 2009;**153**:A768.

2. Flohil SC, de Vries E, Neumann HAM, et al. Incidence, prevalence and future trends of primary basal cell carcinoma in the Netherlands. *Acta Dermatol Venereol* 2010;**90**:24-30.

3. Flohil SC, Koljenović S, de Haas ER et al. Cumulative risks and rates of subsequent basal cell carcinomas in the Netherlands. *Br J Dermatol* 2011;**165**:874-881.

4. De Vijlder HC, Neumann HAM, de Haas ERM. Van klinische studie tot dagelijkse praktijk; Nog een vertaalslag te maken. 2012 (submitted).

5. Nishigori C. Cellular aspects of photocarcinogenesis. *Photochem Photobiol Sci* 2006;**5**:208-214.

6. Scandalios JG. The rise of ROS. *Trends in Biochem Sci* 2002;**27**:483-486.

7. Dutch dermatologic oncology CBO guideline for BCC, 2007.

8. Bath Hextall FJ, Perkins W, Bong J, Williams HC. Interventions for basal cell carcinoma of the skin. *Cochrane database Syst Rev* 2007;**24**:CD003412. Review.

9. Sekulic A, Migden MR, Oro AE et al. Efficacy and safety of vismodegib in advanced basal-cell carcinoma. New Engl J Med. 2012;**366**:2171-2179.

10. Dutch dermatologic oncology CBO guideline for SCC, 2012.

11. Brown SB, Brown EA, Walker I. The present and future role of PDT in cancer treatment. *Lancet Oncol* 2004;**5:**497-508.

12. Fan BG, Andrén-Sandberg A. PDT for pancreatic cancer. *Pancreas* 2007;**34**:385-389.

13. Davidson SR, Weersink RA, Haider MA, et al. Treatment planning and dose analysis for interstitial PDT of prostate cancer. *Phys Med Biol* 2009;**54**:2293-2313.

14. Wildeman MA, Nyst HJ, Karakullukcu B, Tan BI. PDT in the therapy for recurrent/persistent nasopharyngeal cancer. *Head Neck Oncol* 2009;**17**:1-40. Review.

15. Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: update; British Association of Dermatologists Therapy Guidelines and Audit Subcommittee and the British Photodermatology Group. *Br J Dermatol* 2008;**159**:1245-1266.

16. De Visscher SAHJ, Dijkstra PU, Tan IB, et al. mHPTC mediated photodynamic therapy (PDT) of squamous cell carcinoma I the head and neck: A systematic review. *Oral Oncol* 2012 09 011 (Epub ahead of print)

17. Triesscheijn M, Baas P, Schellens JHM, Stewart FA. Photodynamic therapy in oncology. *The Oncologist* 2006;**11**:1034-1044.

18. Busch TM. Local physiological changes during photodynamic therapy. *Lasers Surg Med* 2006;**38**:494-499.

19. Moan J. On the diffusion length of singlet oxygen in cells and tissues. *J Photochem Photobiol B:Biol* 1990;**6**:343-344.

20. Agostinis P, Berg K, Cengel KA et al. Photodynamic therapy of cancer: an update. *CA Cancer J Clin* 2011;**61**:250-281.

21. Fingar VH, Wieman TJ, Wiehle SA, Cerrito PB. The role of microvascular damage in photodynamic therapy: the effect of treatment on vessel constriction, permeability and leucocyte adhesion. *Cancer Res* 1992;**52**:4914-4921.

22. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992;**55**:145-157.

23. Henderson BW, Waldow SM, Mang TS et al. Tumor destruction and kinetics of tumor cell death in two experimental mouse tumors following photodynamic therapy. *Cancer Res* 1985;**45**:572-576.

24. Henderson BW, Fingar VH. Oxygen limitation of direct tumor cell kill during photodynamic treatment of a murine tumor model. *Photochem Photobiol* 1989;**49**:299-304.

25. Bhuvaneswari R, Gan YY, Soo KC, Olivo M. The effect of photodynamic therapy on tumor angiogenesis*. Cell Mol Life Sci* 2009;**66**:2275-2283.

26. De Vree WJA, Essers MC, de Bruijn HS et al Evidence for an important role of neutrophils in the efficacy of photodynamic therpy in vivo. *Cancer research* 1996;**56**:2908-2911.

27. De Bruijn HS, Sluiter W, van der Ploeg-van den Heuvel A et al. Evidence for a bystander role of neutrophils in the response to systemic 5-aminolevulinic acid-based photodynamic therapy. *Photochem Photobiol Photomed* 2006;**22**:238-246.

28. Gollnick SO. Photodynamic therapy and antitumor immunity. J Natl Compr Canc Netw. 2012;**10 Suppl 2**:S40-43.

29. Brackett CM, Gollnick SO. Photodynamic therapy enhancement of anti-tumor immunity. *Photochem Photobiol Sci* 2011;**10**:649–652.

30. Kennedy JC, Pottier RH, Pross DC. PDT with endogenous protoporhyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B* 1990;**6**:143-148.

31. Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitiser for PDT. *J Photochem Photobiol B* 1992;**14**:275-292.

32. Wilson BC, Olivo M, Singh G. Subcellular localisation of photofrin and aminolevulinic acid and cross-resistance in vivo in radiation induced fibrosarcoma cells sensitive or resistant to photofrin-mediated photodynamic therapy. *Photochem Photobiol* 1997;**65:**166-176.

33. Liang H, Shin DS, Eddie Lee Y et al. Subcellular phototoxicity of 5-aminolevulinic acid. *Lasers Surg Med* 1998;**22:**14-24.

34. Uehlinger P, Zellweger M, Magnieres G et al. 5-aminolevulinic acid and its derivatives: physical chemical properties and PpIX formation in cultured cells. *J. Photochem Photobiol B* 2000;**54**:72-80.

35. Datta SN, Loh CS, MacRobert AJ et al. Quantitative studies of the kinetics of 5-aminolaevulinic acid induced fluorescence in bladder transitional cell carcinoma. *Br J Cancer* 1998;78:1113-1118.

36. Biel MA. PDT of head and neck cancers. *Methods Mol Biol* 2010;**635**:281-293.

37. Wang JB, Liu LX. Use of PDT in malignant lesions of stomach, bile duct, pancreas, colon and rectum. *Hepatogastroenterol* 2007;**54**:718-724.

38. Svanberg K, Andersson T, Killander D et al. PDT of nonmelanoma tumours of the skin using topical d-aminolevulinic acid sensitisation and laser irradiation. *J Dermatol* 1994;**130**:743-751.

39. Meijnders PJN, Star WM, de Bruijn HS et al. Clinical results of PDT for superficial skin malignancies and actinic keratosis using topical 5-aminolevulinic acid. *Lasers Med Sci* 1996;**11:**123-131.

40. Wennberg AM, Lindholm LE, Alpsten M, Larko O. Treatment of superficial basal cell carcinomas using topically applied delta-aminolevulinic acid and filtered Xenon lamp. *Arch Dermatol Res* 1996;**11**: 123-131.

41. Peng Q, Soler AM, Warloe T et al. Selective distribution of porphyrins in skin thick basal cell carcinoma after topical application of methyl 5-aminolevulinate *J Photochem Photobiol* 2001;**62**:140-145.

42. Tope WD, Bhardwaj SS. Photodynamic therapy in Dermatology, in Bolognia JL, Jorizzo JL and Rapini RP (Eds.) *Dermatology* 2008:pp2071-2080, Mosby-Elsevier, Spain, 2nd edition.

43. Anand S, Honari G, Hasan T, Elson P, Maytin EV. Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells *in vitro* and *In vivo*. *Clin Cancer Res* 2009;**15**:3333-3343.

44. Anand S, Wilson C, Hasan T, Maytin EV. Vitamin D3 enhances the apoptopic response of epithelial tumors to aminolevulinate-based photodynamic therapy. *Cancer Res* 2011;**71**:6040-6050.

45. Kloek J, Akkermans W, Beijersbergen van Henegouwen GM. Derivatives of 5-aminolevulinic acid for photodynamic therapy: enzymatic conversion into protoporphyrin. *Photochem Photobiol* 1998;**67**:150-154.

46. De Bruijn HS, Meijers C, van der Ploeg-van den Heuvel A, et al. Microscopic localisation of protoporphyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl 5-aminolevulinate. *J Photochem Photobiol B* 2008;**92**:91-97.

47. Togsverd-Bo K, Lerche CM, Philipsen PA et al. Porphyrin biodistribution in UV-exposed murine skin after methyl- and hexyl-aminolevulinate incubation. *Exp Dermatol* 2012;**21**:260-264.

48. Rud E, Gederaas O, Hogset A, Berg K. 5-Aminolevulinic acid, but not 5-aminolevulinic acid esters, is transported into adenocarcinoma cells by system beta transporters. *Photochem Photobiol* 2000;**71**:640-647.

49. Moan J, Ma LW, Juzeniene A et al. Pharmacology of protoporphyrin IX in nude mice after application of ALA and ALA esters. *Int J Cancer* 2003;**103**:132-135.

50. Morton CA, Whitehurst C, Moseley H et al. Comparison of PDT with cryotherapy in the treatment of Bowen's disease. *Br J Dermatol* 1996;**135**:766-771.

51. Haller JC, Cairnduff F, Slack G et al. Routine double treatments of superficial basal cell carcinomas using aminolaevulinic acid-based photodynamic therapy. *Br J Dermatol* 2000;**143***:*1270-1274.

52. van der Veen N, van Leengoed HLLM, Star WM. In-vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 1994;**70**:867-872.

53. Robinson DJ, de Bruijn HS, de Wolf J et al. Topical 5-aminolevulinic acid-photodynamic therapy of hairless mouse skin using two-fold illumination schemes: PpIX fluorescence kinetics, photobleaching and biological effect. *Photochem Photobiol* 2000;**72**:794-802.

54. de Bruijn HS, van der Veen N, Robinson DJ, Star WM. Improvement of systemic 5-aminolevulinic acid-based photodynamic therapy in vivo using light fractionation with a 75-minute interval. *Cancer Research* 1999;**59**:901-904.

55. Robinson DJ, De Bruijn HS, Star WM, Sterenborg HJ. Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated PDTof hairless mouse skin. *Photochem. Photobiol* 2003;**77:**319-323.

56. van der Veen N, Hebeda KM, de Bruijn HS, Star WM. Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single or two-fold illumination. *Photochem Photobiol* 1999;**70**:921-929.

57. De Haas ERM, Kruijt B, Sterenborg HJ, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolaevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

58. Sotiriou EE, Apalla Z, Chovarda E et al. Single vs. fractionated photodynamic therapy for face and scalp actinic keratoses: a randomized, intra-individual comparison trial with 12-month follow-up. *J Eur Acad Dermatol Venereol* 2012;**26**:36-40.

59. van der Veen N, de Bruijn HS, Star WM. Photobleaching during and re-appearance after photodynamic therapy of topical ALA-induced fluorescence in UVB-treated mouse skin. *Int J Cancer* 1997;**72**:110-118.

60. Orenstein A, Kostenich G, Malik Z. The kinetics of protoporphyrin fluorescence during ALA-PDT in human malignant skin tumors. *Cancer Lett* 1997;**120**:229-234.

61. Klintenberg C af, Enejder AMK, Wang I et al. Kinetic fluorescence studies of 5-aminolaevulinic acid-induced protoporphyrin IX accumulation in basal cell carcinomas. *J Photochem Photobiol B* 1999;**49**:120-128.

62.de Bruijn HS, Kruijt B, van der Ploeg–van den Heuvel A et al. Increase in protoporphyrin IX after 5-aminolevulinic based photodynamic therapy is due to local re-synthesis*. Photochem Photobiol Sci* 2007;**6**:857-864.

63.de Bruijn HS, van der Ploeg–van den Heuvel A, Sterenborg HJCM, Robinson DJ. Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice: The influence of the dark interval. *J Photochem Photobiol B:Biol* 2006:**85**;184-190.

64.de Bruijn HS, de Haas ER, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated PDT in normal mouse skin. *Photochem Photobiol Sci.* 2007;**6**:1325-1331.

65. Cottrell WJ, Paquette AD, Keymel KR et al. Irradiance-dependent photobleaching and pain in delta-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res* 2008;**14**:4475-4483.

66. de Bruijn HS, Casas A, Di Venosa G et al. Light fractionated ALA-PDT enhances therapeutic efficacy in-vitro; the influence of PpIX concentration and illumination parameters. *Photochem Photobiol* 2012, Oct 29 [Epub ahead of print].

67. Oleinick NL, Morris RL, Belinchenko I. The role of apoptosis in response to photodynamic therapy: what, where, why and how. *Photchem Photobiol Sci* 2002;**1**:1-21.

68. Kessel D, Vicente MG, Reiners JJ. Initiation of apoptosis and autophagy by photodynamic therapy. *Lasers Surg Med* 2006;**38**:482-488.

69. Buyaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim Biophys Acta* 2007;**1776**:86–107.

70. Ji HT, Chen LT, Lin YH et al. 5-ALA mediated photodynamic therapy induces autophagic cell death via the AMP-activated protein kinase. *Mol Cancer* 2010;**9**:91.

71. Coupienne I, Bontems S, Dewaele M et al. NF-kappaB inhibition improves the sensitivity of human glioblastoma cells to 5-aminolevulinic acid-based photodynamic therapy. *Biochem Pharmacol* 2011;**81**:606-616.

72. Ji Z, Yang G, Vasovic V, Cunderlikova B et al. Subcellular localization pattern of protoporphyrin IX is an important determinant for its photodynamic efficiency of human carcinoma and normal cell lines. *J Photochem Photobiol B* 2006;**84**:213-320.

73. Caekelbergh K, Annemans L, Lambert J et al. Economic evaluation of methyl aminolevulinate-based photodynamic therapy in the management of actinic keratosis and basal cell carcinoma. *Br J Dermatol* 2006;**155:**784-790.

74. Kelleners-Smeets N. Comparative cost minimization study in treatment of superficial basal cell carcinoma: photodynamic therapy versus imiquimod vs fluorouracil, a randomized controlled trial.*www.zonmw.nl/nl/projecten/project-detail/comparative-cost-minimization-study-in-treatment-of-superficial-basal-cell-carcinoma-photodynamic-therapy.*

75. De Haas ERM, de Vijlder HC, van Reesema WS, et al. Quality of clinical practice guidelines in dermatological oncology. *J Eur Acad Dermatol Venereol* 2007;**21**:1193-1198.

76. De Haas ERM, de Vijlder HC, Sterenborg HJCM, et al. Fractionated aminolaevulinic acid-photodynamic therapy provides additional evidence for the use of PDT for non-melanoma skin cancer. *J Eur Acad Dermatol Venereol* 2008;**22**:426-430.

77. De Vijlder HC, Middelburg TA, De Bruijn HS, et al. Optimizing ALA-PDT in the management of non-melanoma skin cancer by fractionated illumination. *G Ital Dermatol Venereol* 2009;**144**:433-439.

2 Light fractionation significantly improves the response of superficial basal cell carcinoma to ALA-PDT: Five-year follow-up of a randomized, prospective trial

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ABSTRACT

Photodynamic therapy (PDT) using topical porphyrin precursors is a promising treatment for superficial basal cell carcinoma (sBCC), but needs further optimization. The aim of the present study was to compare 5-year lesion (complete) response rates of sBCC treated with topical aminolevulinic acid (ALA)-PDT using a single illumination versus ALA-PDT using a 2-fold illumination scheme. A prospective, randomized study was performed in which 91 patients with 299 lesions treated with a 2-fold illumination scheme in which two light fractions of 20 and 80 Jcm^{-2} were delivered 4 and 6 hours after a single application of 20% ALA and 104 patients with 274 lesions treated with a single illumination of 75 Jcm^{-2}, 4 hours after ALA application. All lesions were treated at a fluence rate of 50$mWcm^{-2}$. An interim time to event analysis of complete response (CR) rates at 12 months had shown encouraging results, and therefore lesions were followed for 5 years post therapy. A third group of 50 patients with 172 lesions treated with a 2-fold illumination were included after the initial period and analyzed separately. The CR rate was significantly greater following the 2-fold illumination than the single illumination ($p = 0.0002$, log-rank test). Five years after therapy the CR rate after a 2-fold illumination was 88% whereas the CR rate to single illumination is 75%. The CR rate in the third group of lesions, treated with 2-fold illumination was 97% and 88% at 12 months and 5 years after therapy, respectively. Long-term follow-up indicates superior efficacy of ALA-PDT with a 2-fold illumination scheme in superficial basal cell carcinoma compared to ALA-PDT with single illumination.

INTRODUCTION

Photodynamic therapy (PDT) is under development as an experimental therapy for many tumour types.[1] Its effectiveness as a curative or palliative treatment in certain niche clinical applications is well documented.[2-4] Treatment consists of two relatively simple procedures: the administration of a photosensitive drug or pre-cursor and the illumination of the tumour with visible light. This leads to the generation of reactive oxygen species, notably singlet oxygen[5] and results in the destruction of the tumour by a combination of direct cellular and secondary vascular effects.[6] In addition PDT is able to initiate an immune response against the remaining tumour cells.[7] A successful outcome following PDT is reliant on each of these mechanisms and the relative contribution of each depends on the photosensitizer and treatment regimen.[8] Except for temporary skin photosensitization, there are no long-term side effects if appropriate protocols are followed. Healing occurs with little or no scarring and the procedure can be repeated without cumulative toxicity. The main drawback against using PDT as frontline therapy lies in the fact that PDT has generally only been tested in small scale studies, long term clinical response studies and large randomized trials are rare.[1]

One approach to PDT is the use of porphyrin pre-cursors such as aminolaevulinic acid (ALA) which has been under investigation for over 2 decades.[9,10] ALA is itself not a photosensitizer but a precursor of protoporphyrin IX (PpIX). ALA is converted within cells into PpIX, via the heme cycle. ALA-PDT has been used topically for the treatment of non-melanoma skin cancer (NMSC)[11] and orally for cancer in the oral cavity[12] and gastrointestinal tract.[13] The advantage of topical ALA-PDT over other types of PDT using pre-formed photosensitizers is the absence of widespread skin photosensitization, except for the application area. The short term efficacy for topical ALA-PDT in NMSC has been demonstrated in many clinical trials (reviewed in[11]) and ALA and one of its derivatives are now approved for clinical use.[14]

While ALA-PDT provides good short term results, long term clinical results are sparse and much less impressive and show a considerable variation in recurrence rates.[15] This has prompted investigators to search for approaches to improve the response to PDT. A number of factors limit response. While the most important of these is the rapid photobleaching of PpIX during illumination which limits the maximum PDT dose that can be delivered,[16] other factors play a role: The availability of ALA to cells in deeper regions of the skin lesions may be limited by the penetration of topically applied ALA.[17] PpIX accumulation may be limited by the capacity of the heme synthesis pathway. The response to PDT can be limited by the availability of oxygen and the distribution of light in tissue.[18] The uptake of ALA and/or the accumulation of PpIX can be improved by the use of iontopheresis,[19] penetration enhancers[20] or iron chelators.[21] Moreover several alkyl esters of ALA have been developed with the intention of enhancing cell uptake and tissue penetration.[22] Methyl aminolaevulinate

(Metvix®) was approved in the European Union in 2001 for the treatment of actinic keratosis (AK) and basal cell carcinoma (BCC).[23] Given these efforts to enhance PDT with porphyrin pre-cursors it is disappointing that few large-scale, long-term clinical studies have been performed to investigate whether these approaches are effective. In the small number of case studies that exist results have been disappointing.[24]

We have been investigating an approach to enhance the response following ALA-PDT by changing the illumination parameters.[25] We have performed numerous pre-clinical studies investigating the effect of splitting the illumination into two light fractions separated by a dark interval of several hours. We have shown that the response to this type of light fractionated approach is enhanced over a single illumination[26] and that this increase is largest when a low-dose light fraction followed by a high-dose light fraction is applied, separated by a dark interval of two hours. We have shown that the choice of fluence (rate) for the first fraction is critical, and a high fluence for the second light fraction and a two-hour dark interval is necessary for maximal tissue response.[27] Based on these preclinical results a randomized comparative prospective open clinical study was performed in the treatment of superficial basal cell carcinoma (sBCC), comparing traditional non-fractionated ALA-PDT using a single fluence of 75 Jcm^{-2} and a 2-fold illumination of 20 + 80 Jcm^{-2}.[28] The choice of a single dose of 75 Jcm^{-2} compared to the cumulative dose of 100 Jcm^{-2} in the fractionated group is based on: (*i*) the fact that photobleaching of PpIX limits the PDT dose that can be delivered in a single fraction at fixed fluence rate[16] and (*ii*) our findings that a high fluence second light fraction is most effective.[26] An interim analysis of CR rate time-to-event analysis at 12 month showed a significantly better result for fractionated PDT.[28] In the present study we report on the 5 year follow-up data analysis of our randomized clinical trial.

MATERIALS AND METHODS

Patients

The study design and clinical procedure have been described in more detail previously.[28] Briefly, all patients were diagnosed as having a sBCC within the department of dermatology of the Erasmus MC in Rotterdam. ALA-PDT was performed from July 2002 to November 2004 according to 2 treatments protocols, approved by the local ethics committee, in accordance to the principles of the Declaration of Helsinki. All patients gave informed consent. A total of 104 patients who altogether had in total 274 lesions, were treated using a single illumination scheme and 91 patients with in total 299 lesions, using a 2-fold illumination scheme as summarized in figure 1.

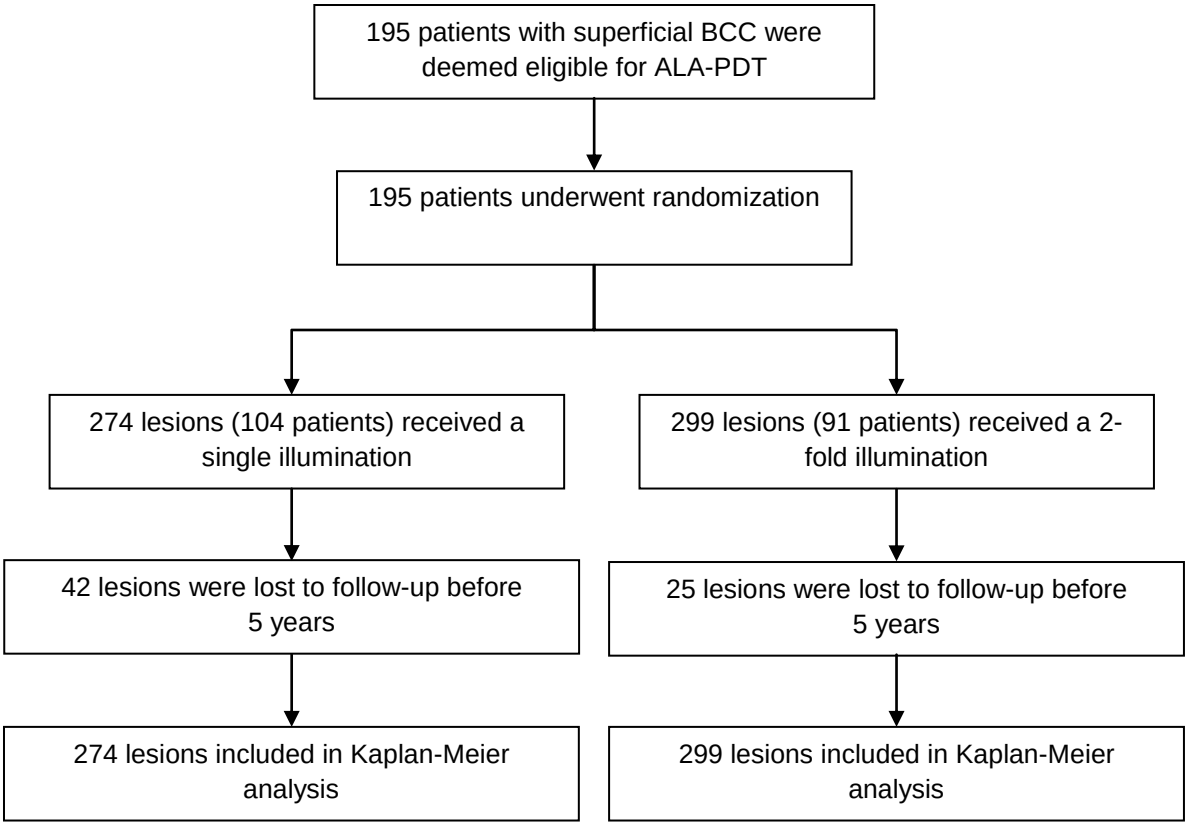

Figure 1: Flow diagram of patient and lesion inclusion, allocation, follow-up, and data analysis of patients undergoing ALA-PDT using a single and a 2-fold illumination scheme

A 12 months interim analysis of these two groups of patients resulted in a statistically significant increase in CR rate in the group receiving the 2-fold illumination. Given this result, a third group of 50 additional patients with 172 lesions that received only the 2-fold illumination were included between November 2004 and August 2005. All patients were followed for a period of 5 years. Diagnosis was determined histologically and clinically in approximately equal proportions within all 3 treatment groups. Patient characteristics in the 3 groups were comparable: All patients were adult Caucasians with a mean age of 56.7 years (range 31-88) in the single illumination group, a mean age of 56.9 years (range 32-84) in the group receiving a 2-fold illumination scheme from July 2002 to November 2004, and 65.5 years (range 39-90) in the third group receiving a 2-fold illumination scheme from November 2004 to August 2005. The number of high-risk patients in each treatment group is shown in Table 1.

Table 1: High-risk patients for developing skin cancer

Patient group	75 Jcm^{-2}		20+ 80 Jcm^{-2}		20+ 80 Jcm^{-2}	
	Patients n	Lesions n(%)	Patients n	Lesions n(%)	Patients n	Lesions n(%)
Immuno-compromised [a]	5	5 (1.8)	4	16 (5.3)	2	2 (1.2)
Previous radiotherapy	6	29 (10.6)	5	16 (5.4)	4	6 (3.5)
Goltz Gorlin syndrome	5	46 (16.7)	5	31 (10.4)	3	33 (19.2)
High sun exposure [b]	3	18 (6.6)	3	29 (9.7)	2	10 (5.8)
Total high-risk	18	96 (35.0)	17	92 (30.8)	11	51 (29.7)

[a] Immuno-compromised: HIV positive, organ recipient, or using immunosuppressive drugs
[b] Patients who have lived more than 15 years in tropical countries and had Fitzpatrick skin type 1.

Aminolaevulinic acid application and light sources

The topical ALA ointment used was prepared by our hospital pharmacy using 20% ALA (FLUKA, Zwijndrecht, The Netherlands) in Instilagel® (Medeco BV, Oud Beijerland, The Netherlands). The ointment was freshly prepared, stored in refrigerator and used within 3 days. Before application of ALA ointment, crusts and scaling were gently removed using a disposable curette. Thereafter the lesion was covered with a margin of 1 cm and dressed with a semi permeable dressing (Tegaderm 3M, The Netherlands) and light-protecting dressing (aluminum foil).

The light sources used in this study were a 630 nm diode laser (Carl Zeiss, Oberkochen, Germany) and two broadband light sources: a broadband light source with a spectral output between 590 and 650 nm (Medeikonos, Gothenburg, Sweden) and a light-emitting diode (LED) light source with a spectral output centred on 633 nm with a bandwidth of 20 nm (Omnilux, Waldman, Tiel, The Netherlands). All 3 light sources were used to illuminate lesions with a margin of at least 5 mm at a constant measured fluence rate of 50 $mWcm^{-2}$. In the single illumination group, lesions were illuminated 4 h after the application of ALA ointment to a fluence of 75 Jcm^{-2}. In both 2-fold illumination groups, lesions received light fractions of 20 and 80 Jcm^{-2}, 4 and 6 h after the application of ALA ointment. ALA ointment was applied once. Both light fractions were delivered at a fluence rate of 50 $mWcm^{-2}$. During the two hour interval between the light fractions, lesions were covered with light-protective dressing.

Response and follow-up

Patients were followed up during the 5 year interval between treatment and a final follow-up assessment between April 2009 and August 2010. The overwhelming majority of patients were seen in repeat visits to our department. Files of patients who were originally referred to our department for treatment in connection with our study and referred back to their peripheral primary dermatologist, were examined if necessary at our department. Out of a total of 245 patients, 31 were lost to follow-up of whom 18 patients died. Follow-up was performed by staff members and residents within our department. A small number of patients were seen by peripheral dermatologists. One investigator (HdV) was responsible for the final clinical follow-up and was blinded for the treatment scheme delivered to each lesion.

Complete response (CR) was defined as the absence of clinically visible BCC. Histologically confirmed recurrences were identified via patient files and digital photographs. If lesions, histologically confirmed, occurred at the same body area as the original lesion, and no detailed report or/and photographs were present, they were considered as recurrences (R). Lesions in patients who were lost to follow-up, due for any reason were included appropriately in our statistical analysis. Recurrences and lesions with a partial response were retreated with either fractionated PDT or surgical excision and were not included in our statistical analysis.

Statistics

Kaplan-Meier analysis was performed on relative CR rates after therapy and the log-rank test was used to compare significance of differences between the non-fractionated and fractioned groups included before November 2004 and to check the consistency of the data of the fractioned group, included thereafter. Data was right censored if lesions were lost to follow-up or were lost to follow-up because of the death. A secondary Kaplan-Meier analysis was performed assuming that lesions lost to follow-up had recurred at their time of last observation. Primary and overall response rates of lesions treated with different illumination schemes at specific time intervals after therapy were compared using Fisher's exact test.

RESULTS

2-fold illumination of 20+80 Jcm^{-2} with a 2-h dark interval results in a significantly better clinical response to aminolaevulinic acid-photodynamic therapy compared with a single light fraction.

The relative CR of lesions following ALA-PDT using a single light fraction and a 2-fold illumination scheme is shown is figure 2. The relative CR using a 2-fold illumination scheme was significantly greater than that following a single light fraction ($p = 0.0002$, log rank test).

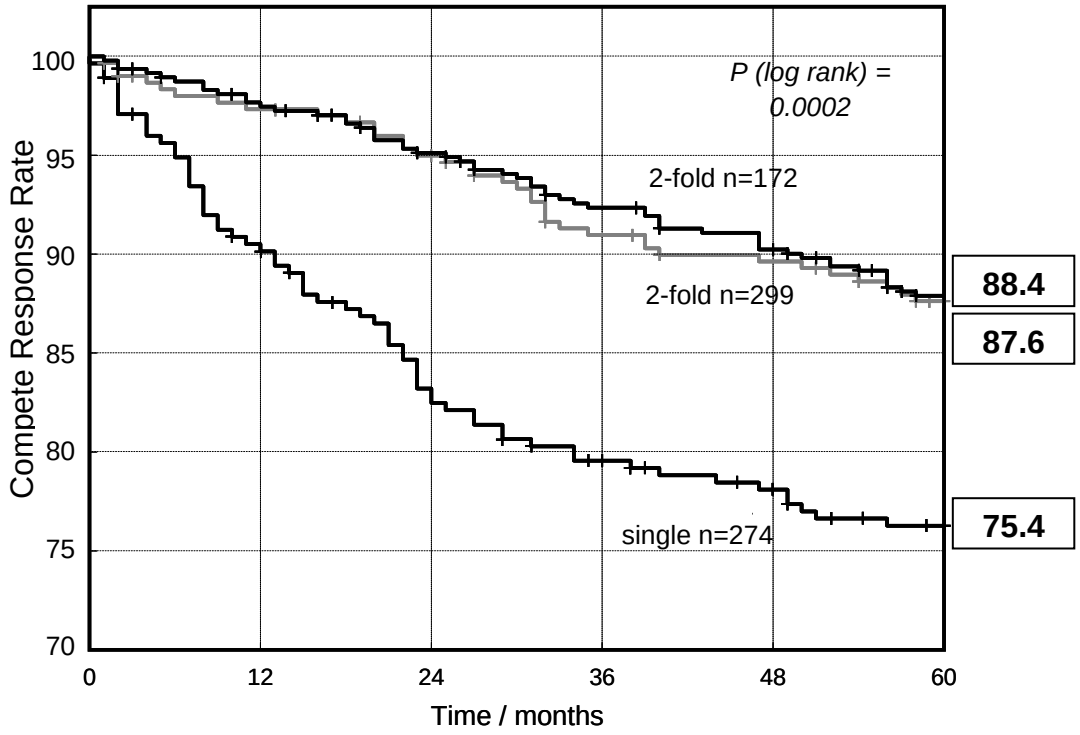

Figure 2: Kaplan-Meier curves for complete response rate of sBCC lesions using a single light fraction of 75 J cm^{-2}, 4 hours after the application of ALA (bottom black curve, n = 274) and a 2-fold illumination scheme of the first group of 20+80 Jcm^{-2}, (grey curve; n = 299) 4 and 6 hours after the application of ALA. The top black curve shows a second subsequent group of lesions treated with a 2-fold illumination scheme; n = 172. Note: the ordinate, complete response rate, is plotted from 70-100% to aid visualization.

Table 2: Outcome of 745 lesions; complete response (CR), recurrence, partial response, loss to follow-up at 5 years. A. Prospective randomized lesions, B. Subsequently treated lesions

Patient group	75 Jcm^{-2} n=274 n(%)	20+ 80 Jcm^{-2} (A) n=299 n(%)	20+ 80 Jcm^{-2} (B) n=172 n(%)
Complete response	165 (60.2)	237 (79.3)	139 (80.8)
Recurrence	52 (19.0)	31 (10.4)	20 (11.6)
Partial response	15 (5.5)	6 (2.0)	0 (0)
Died	27 (9.9)	17 (5.7)	2 (1.2)
Lost to follow-up	15 (5.5)	8 (2.7)	11 (6.4)

Five years after therapy, the relative CR in the 2-fold illumination group was 88%, whereas the corresponding CR in the single illumination group was 75% (p = 0.0002, Fisher exact test). The relative CR in the second fractionated group was also 88% which confirmed the result of the first fractionated group. Of the 274 lesions in the single illumination group 15 failed to respond and 52 recurred during the follow-up period. Of the 299 lesions in the 2-fold illumination group, 6 failed to respond and 31 recurred during follow-up period. Of the 172

lesions in the second 2-fold illumination group, no lesions failed to respond and 20 recurred during follow-up period (Table 2).

A secondary Kaplan-Meier analysis which assumed lesions lost to follow-up had recurred at the time of their last observation also showed a very significant difference in CR between the single and 2-fold illumination scheme ($p = 0.0002$, log rank test). In this analysis the relative CR 5 years after therapy in the 2-fold illumination group was 79%, whereas the corresponding CR in the single illumination group was 60% ($p = 0.0002$, Fisher's exact test).

Subgroup analysis

A subgroup analysis for high-risk patients (Gorlin-Goltz syndrome (nevoid basal cell carcinoma syndrome (OMIM #109400)), immune-compromised, prior radiotherapy, high sun exposure, and arsenic injection exposure) was performed for each treatment group. Patients in each of these high-risk groups did not influence the relative CR rates of the group receiving single illumination or the groups receiving 2-fold illumination.

A subgroup analysis for the different light sources used (630 nm diode laser, broadband light source; spectral output 590 - 650 nm and a LED light source; spectral output 613 – 633 nm), did not influence the relative CR observed between treatment schemes.

A subgroup analysis for body site showed that lesions defined as treated within the hairy scalp showed significantly more recurrences compared with other body sites in both illumination groups ($p < 0.01$, log rank test, in each case). In the single illumination group 71% (5 lesions of a total of 7 lesions) located at this location showed recurrence whereas in the combined fractionated group 55% (5 lesions of a total of 9 lesions) recurred. There was no statistically significant difference in the response rate between lesions treated within the hairy scalp using the single or 2-fold illumination.

DISCUSSION

The aim of the study was to determine whether our attempts to enhance ALA-PDT *in vivo* using light fractionation results in a clinically significant increase in the long-term response of sBCC. We have previously shown that performing light fractionated PDT is significantly more effective in pre-clinical models.[26,27,29-31] Based on these results a large randomized comparative prospective open clinical study was performed in the treatment of 573 superficial BCC lesions, comparing ALA-PDT using one single fluence of 75 Jcm^{-2} and a 2-fold illumination of 20 + 80 Jcm^{-2}.[28] An interim analysis at 12 months demonstrated a statistically significant improvement in CR rate for light fractionated PDT (97% vs 89% CR, $p <0.002$, log-rank test). The data we present in the present study demonstrates that this statistically significant improvement in CR rate is maintained after 5 years follow-up (88% vs

75% CR, p = 0.0002, log-rank test). This study is one of the few large scale long-term randomized clinical studies investigating PDT response and the first to show that efforts to optimize PDT can lead to a significant increase in long-term clinical response. This increase in clinical response is not simply a statistical difference; it is a significant result for patients. Light fractionated ALA-PDT requires approximately 1 in 10 patients to be retreated after 5 years compared with 1 in 4 using the traditional approach to performing ALA-PDT.

Comparing our current results with those of small-scale short-term studies in the literature using ALA-PDT for sBCC[11,15] we conclude that the CR rates for light fractionated PDT are much higher at all time points while the CR rates of our group treated with single illumination correspond well with those reported by others using ALA-PDT.[11, 32] For MAL-PDT longer and more extensive follow-up data are available; for example Basset et al.[33] performed a randomized clinical trial comparing MAL-PDT (1-2 treatments) with cryotherapy (1-2 treatments) with a follow up of 5 years in 219 sBCC where 114 lesions received MAL-PDT. A recurrence rate of 22% was reported at 5 years with one or two PDT treatment sessions. It is difficult to compare recurrence rates exactly, since the rates are based on the total number of patients with recurrent BCC divided by the number of patients with initial tumours treated. This method ignores the patients who are unavailable for follow-up and artificially lowers the recurrence rates reported. In CR rates calculated by the Kaplan-Meier survival response all information about survival and lost to follow-up are included. However keeping these caveats in mind, recurrence rates of ~11% in our study appear to be significantly better than the 22% recurrence rates reported after conventional MAL-PDT. Therefore, in addition to the increase in CR rate using our light-fractionated approach patients do not require a second treatment day as recommended by the registered protocol for MAL-PDT using Metvix®. In healthcare decision making, economic arguments need to be considered alongside clinical efficacy. Fractionated ALA-PDT appears to be significantly more cost-effective than MAL-PDT. ALA in Instillagel® is less expensive than Metvix® and our approach requires only a single treatment day. This reduces both direct and indirect costs and the (psychological) burden for the patient. Interestingly the CR rate of our group treated with a single illumination is similar to that reported for MAL-PDT that is repeated twice.

Surgical excision is generally considered as the treatment of first choice for sBCC. Cumulative recurrence rates after 5 years follow-up reported for surgery vary from 92% to 96.8%, regardless of histological subtype of BCC.[33] Although lower, a 5 year CR of 88% observed in our group treated with fractionated ALA-PDT approaches the CR rate reported for surgery. Importantly, a generally better cosmetic outcome is reported after PDT in the treatment of sBBC compared with other treatment modalities, such as surgery and cryotherapy.[34] It is encouraging that our study showed that the cosmetic outcome after one

year follow-up was good to excellent for both single and light-fractionated groups.[27] In addition to good cosmesis PDT is associated with minimal skin deterioration which is a drawback of invasive surgery: The risk of disfigurement, loss of function, or delayed wound healing at sites with poor vasculature and the risk of postsurgical bleeding and scarring is relatively low with PDT. A disadvantage of PDT is pain during illumination. A minority of our patients (14%) required additional pain relief (2% lidocaine subcutaneously). After the illumination pain resolved quickly and there was no statistical difference in pain experience between the non-fractionated and fractionated group.

We performed a subgroup analysis of patients with a higher risk of developing skin cancer (Goltz Gorlin syndrome, immunocompromised, prior radiotherapy, high sun exposure, and arsenic exposure) and found that these patients did not show significantly different response rates in either illumination group. No difference in increased efficacy was found in patients with multiple lesions or those with sporadic sBCC. This result supports data that show the efficacy of MAL-PDT in patients with Goltz-Gorlin syndrome or after radiotherapy are comparable with sporadic BCCs.[35]

There are several factors known to affect the recurrence rate of BCC. The scalp is regarded as relatively high risk localization for recurrences.[36,37] A subgroup analysis in the present study confirmed that lesions in the hairy scalp showed relatively more recurrences compared with other body sites in all three treatment groups: a CR 45% vs. 88% in the fractionated group and 29% vs. 75% in the non-fractionated group. Based on these results our conclusion is that ALA-PDT is a suboptimal treatment for sBCC on the hairy scalp.

PDT using porphyrin pre-cursors has been applied as an experimental therapy for the treatment of tumour types outside the skin, and its effectiveness is well documented. It has been applied in the oral cavity,[12] in the genitourinary tract[38] and in the gastrointestinal tract.[13] In the latter case the treatment of high grade dysplasia in Barrett's oesophagus has been extensively investigated.[39] A light fractionated approach using ALA-PDT could be applied in other organs, although practical and logistical barriers may be more significant than in skin.

It is important to note that the cumulative fluence in each of our treatment groups is not equal. A fluence of 75 Jcm^{-2} was delivered in a single light fraction compared with 100 Jcm^{-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.[26] The PDT dose delivered in a single light fraction is not directly related to fluence, particularly when the photosensitizer photobleaches rapidly. While the relationship between response to ALA-PDT and fluence has not been systematically investigated in the clinic most investigators have applied both lower light fluence(rate) and light fluences both below 75 Jcm^{-2} and above 100 Jcm^{-2}, with little evidence for a correlation between fluence and response. In retrospect, given our most recent pre-

clinical results[31] we could have chosen a fluence for the second light fraction to be greater than 80 Jcm^{-2}. We note, however, that this choice increases the overall treatment time.

Light fractionation is not the only method that has been studied to enhance the efficacy of PDT using porphyrin precursors. However modulating the delivery of light is a relatively simple practical approach that is easily achievable in a clinical setting. Some investigators have suggested that there may be differences in response to PDT with light sources that deliver a different effective fluence rate by virtue of the overlap of their spectral output with the absorption spectrum of PpIX[40] and light sources that additionally excite the fluorescent photoproducts of PpIX.[41] The fact that we did not see differences in response between light sources suggest that these effects are small and do not impact significantly on the effective dose of the first light fraction. This means that current fluence and dark interval used in the present study are also applicable for the large proportion of investigators that use non-laser light sources.

It is interesting to consider the mechanism behind the increase in efficacy that we have observed in a range of models and speculate whether this mechanism can be utilized to further enhance efficacy. We initially believed that the increased effect was simply a consequence of the additional utilization of PpIX during two light fractions. We have shown in animal models that the effect is somewhat more complex: approximately the same overall amount of PpIX is utilized in treatment schemes that result in significantly different efficacies.[25,26] We have shown using the surrogate metric of monitoring of PpIX photobleaching during therapy that oxygen recovery during the dark interval between light fractions and its utilization during illumination are not significant factors in the increase in response. We have shown that a local immune response is significantly enhanced using light fractionated ALA-PDT but that it is not involved in the mechanism underlying the increase in response.[42] Work is underway to investigate light fractionation effects in cells *in vitro*. We have found that while it is difficult to replicate effects that are observed *in vivo,*[43] preliminary data suggest that efficacy may be enhanced particularly in cells that are sensitized with low levels of PpIX.[44] Also interestingly, we have shown that light fractionation using MAL-PDT does not result in an increase in efficacy in the same way that it does using ALA. In pre-clinical models light fractionated ALA-PDT is associated with significantly more oedema than MAL-PDT.[45] We believe that the microscopic localization of PpIX after application of ALA plays a central role in the increase in response and that vasculature of normal and lesional skin may be a target for the effect that leads to the increase in efficacy. When these mechanisms are fully understood it may be possible to further enhance clinical efficacy and long-term response rates of PDT using ALA and other porphyrin precursors. Encouragingly,

a recent randomized study suggests that our approach to using light-fractionated PDT results in a significantly enhanced clinical response in actinic keratoses.[46]

In conclusion we have demonstrated a significant increase in CR rate of sBBC following ALA-PDT using an illumination scheme in which 2 light fractions of 20 and 80 Jcm^{-2} are delivered 4 and 6 hours after the application of ALA, compared to a traditional illumination.

CONFLICT OF INTEREST

The authors declare no conflicts of interest

REFERENCES

1. Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. *Lancet Oncol* 2004;**5**:497-508.

2. Fan BG, Andrén-Sandberg A. Photodynamic therapy for pancreatic cancer. *Pancreas* 2007;**34**:385-389.

3. Davidson SR, Weersink RA, Haider MA, et al. Treatment planning and dose analysis for interstitial photodynamic therapy of prostate cancer. *Phys Med Biol* 2009;**54**:2293-2313.

4. Wildeman MA, Nyst HJ, Karakullukcu B, Tan BI. Photodynamic therapy in the therapy for recurrent/persistent nasopharyngeal cancer. *Head Neck Oncol* 2009;**1**:40.

5. Weishaupt, R., Gomer CJ, Dougherty TJ. Identification of singlet oxygen as the cytotoxic agent in photo-activation of a murine tumour. *J Natl Cancer Inst* 1993;**85**:443-456.

6. Busch TM. Local physiological changes during photodynamic therapy. *Lasers Surg Med* 2006;**38**:494-499.

7. Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer* 2006;**6**:535-545.

8. Woodhams JH, Macrobert AJ, Bown SG. The role of oxygen monitoring during photodynamic therapy and its potential for treatment dosimetry. *Photochem Photobiol Sci* 2007;**6**:1246-1256.

9. Kennedy JC, Pottier RH, Pross DC. Photodynamic therapy with endogenous protoporhyrin IX: basic principles and present clinical experience. *J Photochem Photobiol B* 990;**6:**143-148.

10. Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. *J Photochem Photobiol B* 1992;**14**:275-292.

11. Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: update; British Association of Dermatologists Therapy Guidelines and Audit Subcommittee and the British Photodermatology Group. *Br J Dermatol* 2008;**159**:1245-1266.

12. Biel MA. Photodynamic therapy of head and neck cancers. *Methods Mol Biol* 2010;**635**:281-293.

13. Wang JB, Liu LX. Use of photodynamic therapy in malignant lesions of stomach, bile duct, pancreas, colon and rectum. *Hepatogastroenterology* 2007;**54**:718-724.

14. Lehmann P. Methyl aminolaevulinate-photodynamic therapy: a review of clinical trials in the treatment of actinic keratoses and nonmelanoma skin cancer. *Br J Dermatol* 2007;**156**:793–801.

15. Pariser DM, Lowe NJ, Stewart DM, et al. Photodynamic therapy with topical methyl aminolevulinate (Metvix®) is effective and safe in the treatment of actinic keratosis: results of a prospective randomized trial. *J Am Acad Dermatol* 2003;**48**:227-232.

16. Robinson DJ, de Bruijn HS, van der Veen N, et al. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. *Photochem Photobiol* 1998;**67**:140-149.

17. Peng Q, Soler A-M, Warloe T, et al. Selective distribution of porphyrins in skin thick basal cell carcinoma after topical application of methyl 5-aminolevulinate. *J Photochem Photobiol B: Biol* 2001;**62**:140-145.

18. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992;**55**:145-157.

19. Merclin N, Bramer T, Edsman K. Iontophoretic delivery of 5-aminolevulinic acid and its methyl ester using a carbopol gel as vehicle. *J Control Release* 2004;**98**:57-65.

20. Gerritsen MJ, Smits T, Kleinpenning MM, et al. Pretreatment to enhance protoporphyrin IX accumulation in photodynamic therapy. *Dermatology* 2009;**218**:193-202.

21. Campbell SM, Morton CA, Alyahya R, et al. Clinical investigation of the novel iron-chelating agent, CP94, to enhance topical photodynamic therapy of nodular basal cell carcinoma. *Br J Dermatol* 2008;**159**:387-393.

22. Fotinos N, Campo MA, Popowycz F, et al. 5-Aminolevulinic acid derivatives in photomedicine: Characteristics, application and perspectives. *Photochem Photobiol* 2006;**82**:994-1015.

23. Braathen LR, Szeimies RM, Basset-Seguin N, et al. Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: an international consensus. International Society for Photodynamic Therapy in Dermatology 2005. *J Am Acad Dermatol* 2007;**56**:125-143.

24. Souza CS, Felicio LB, Ferreira J, et al. Long-term follow-up of topical 5-aminolaevulinic acid photodynamic therapy diode laser single session for non-melanoma skin cancer. *Photodiagnosis Photodyn Ther* 2009;**3/4**:207-213.

25. Star WM, van't Veen AJ, Robinson DJ, et al. Topical 5-aminolevulinic acid mediated photodynamic therapy of superficial basal cell carcinoma using two light-fractions with a two-hour interval: long-term follow-up. *Acta Derm Venereol* 2006;**86**:412-417.

26. Robinson DJ, de Bruijn HS, Star WM, Sterenborg HJCM. Dose and timing of the first light fraction in 2-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. *Photochem Photobiol* 2003;**77**:319-323.

27. de Bruijn HS, van der Ploeg-van den Heuvel A, Sterenborg HJ, Robinson DJ. Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice: The influence of the dark interval. *J Photochem Photobiol B* 2006;**85**:184-190.

28. de Haas ERM, Kruijt B, Sterenborg HJCM, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

29. van der Veen N, van Leengoed HL, Star WM. In vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 1994;**70**:867-872.

30. van der Veen N, Hebeda KM, de Bruijn HS, Star WM. Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single- or 2-fold illumination. *Photochem Photobiol* 1999;**70**:921-929.

31. Middelburg TA, Van Zaane F, De Bruijn HS, et al. Fractionated illumination at low fluence rate photodynamic therapy in mice. *Photochem Photobiol* 2010;**86**:1140-1146.

32. Smits T, Moor AC. New aspects in photodynamic therapy of actinic keratoses. *J Photochem Photobiol B* 2009;**96**:159-169.

33. Basset-Seguin N, Ibbotson SH, Emtestam L, et al. Topical methyl aminolaevulinate photodynamic therapy versus cryotherapy for superficial basal cell carcinoma: a 5 year randomized trial. *Eur J Dermatol* 2008;**18**:547-553.

34. Thissen MR, Neumann MH, Schouten LJ. A systematic review of treatment modalities for primary basal cell carcinomas. *Arch Dermatol* 1999;**135**:1177-1183.

35. Mougel F, Debarbieux S, Ronger-Savlé S, et al. Methylaminolaevulinate photodynamic therapy in patients with multiple basal cell carcinomas in the setting of Gorlin-Goltz syndrome or after radiotherapy. *Dermatology* 2009;**219**:138-142.

36. Bøgelund FS, Philipsen PA, Gniadecki R. Factors affecting the recurrence rate of basal cell carcinoma. *Acta Derm Venereol* 2007;**87**:330-334.

37. Telfer NR, Colver GB, Bowers PW. Guidelines for the management of basal cell carcinoma. British Association of Dermatologists. *Br J Dermatol* 1999;**141**:415-423.

38. Soergel P, Hillemanns P. Photodynamic therapy for intraepithelial neoplasia of the lower genital tract. *Photodiagnosis Photodyn Ther* 2010;**7**:10-14.

39. Kelty CJ, Ackroyd R, Brown NJ, et al. Comparison of high- vs. low-dose 5-aminolevulinic acid for photodynamic therapy of Barrett's esophagus. *Surg Endosc* 2004;**18**:452-458.

40. Clark C, Bryden A, Dawe R, et al. Topical 5-aminolaevulinic acid photodynamic therapy for cutaneous lesions: outcome and comparison of light sources. *Photodermatol Photoimmunol Photomed* 2003;**9**:134-141.

41. Gudgin Dickson EF, Pottier RH. On the role of protoporphyrin IX photoproducts in photodynamic therapy. *J Photochem Photobiol B*, 1995;**29**:91-93.

42. de Bruijn HS, Sluiter W, van der Ploeg-van den Heuvel A, et al. Evidence for a bystander role of neutrophils in the response to systemic 5-aminolevulinic acid-based photodynamic therapy. *Photodermatol Photoimmunol Photomed 2006*;**22**:238-246.

43. de Bruijn HS, Light Fractionated ALA-PDT. Thesis, (PhD). Erasmus University Rotterdam 2008.

44. de Bruijn HS, Casas A, Di Venosa G, Rodriguez et al. Light Fractionated ALA-PDT in PAM212 cells. *Photochem Photobiol* 2012, Oct 29 [Epub ahead of print].

45. de Bruijn HS, de Haas ERM, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal Mouse skin. *Photochem Photobiol Sci* 2007;**6**:1325-1331.

46. Sotiriou E, Apalla Z. Chovarda E, et al. Single vs. fractionated photodynamic therapy for face and scalp actinic keratoses: a randomized, intra-individual comparison trial with 12-month follow-up. *J Eur Acad Dermatol* 2012;26:36-40.

3 Differences in protoporphyrin IX localisation in dermal vasculature after topical application of 5-aminolaevulinic acid and methyl amino-laevulinate

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ABSTRACT

We have previously shown significant differences in the response of tissues to light fractionated PDT using the porphyrin precursors 5-aminolaevulinic acid (ALA) and methyl aminolaevulinate (MAL). We were unable to increase the efficacy of MAL-PDT using a two-fold illumination scheme with a 2 hour dark interval optimised for ALA, characterised by significantly more oedema. We present an investigation of the distribution of the photosensitiser PpIX in the dermal vasculature of normal mouse skin 4 hours after the administration of ALA and MAL using fluorophore-conjugated anti-CD31 immunohisto-chemical staining and confocal fluorescence microscopy. We found a significantly higher degree of co-localisation of PpIX and the vasculature after ALA application compared to MAL ($p<0.01$) and low levels of PpIX in the vasculature after MAL application. These results are the first direct indication that the distribution of PpIX in the vasculature is different for MAL and ALA and suggests that endothelial cells are involved in the difference of tissue response to MAL- and ALA-PDT.

INTRODUCTION

We previously reported on the difference in response to light fractionated photodynamic therapy (PDT) between methyl-aminolaevulinate (MAL) and 5-aminolaevulinic acid (ALA).[1] We were unable to increase the efficacy of PDT using MAL simply by using a two-fold illumination scheme with a 2 hour dark interval optimised for ALA.[2-6] A thorough investigation of the differences between ALA and MAL localisation may allow us to fully elucidate the mechanism behind the response of cells and tissue to light fractionation. Previously we have found no significant differences in dermal PpIX concentration and PpIX re-synthesis during the dark interval for ALA and MAL.[1] Differences in the kinetics of PpIX photobleaching, measured at the surface of the skin 4 and 6 hours after the application of ALA or MAL, were observed. PpIX in animals applied with ALA photobleached at a faster rate compared to MAL but these differences were small.[1] In a recent study we investigated the differences in the acute histological response following PDT with MAL - or ALA. Significantly more oedema formation was found after ALA-PDT that was not accompanied by a different inflammatory response to PDT.[7] These observations combined with the well known differences between the pharmacokinetics of ALA and MAL(i.e. the prolonged application of ALA results in the systemic distribution of PpIX whereas PpIX is restricted to the site of MAL application[8]) led us to the conclusion that the vasculature in the dermis may be involved in the difference in response of normal mouse skin following ALA and MAL-PDT. In the same study[7] we aimed to investigate differences in the microscopic distribution of PpIX after topical ALA or MAL administration. PpIX fluorescence in normal mouse skin is dominated by the signal from the

epidermis and stratum corneum. While we were able to find areas of PpIX fluorescence in the dermis, corresponding to the cytoplasm of dermal cells such as fibroblasts and mast cells, we were unable to find any differences in PpIX distribution after the application of either porphyrin precursor. Given these results, in the present study we have investigated differences in tissue distribution of PpIX in more detail. We determined the degree of co-localisation of PpIX with the endothelial marker CD31 using confocal microscopy in frozen sections of tissue recovered from mice applied with topical MAL and ALA. Since this approach is likely very sensitive to small changes in the architecture of biopsy sections and since PpIX fluorescence and CD31 are normally imaged in frozen and fixed samples respectively we have used an adapted immunohistochemical staining procedure in freeze-thawed-frozen sections that combines PpIX fluorescence with CD31-AlexaFluor® 488 imaging. Two wavelength excitation and dual fluorescence band imaging are combined to perform a co-localisation analysis on multiple individual sections of control skin and skin applied with MAL or ALA.

MATERIALS AND METHODS

Animal model and porphyrin precursor application

The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol. We investigated the localisation of PpIX within tissue after topical application with MAL and ALA for 4 hours in three groups of female inbred albino hairless mice (SKH1 hr, Charles River, Someren, The Netherlands), aged between 8 and 10 weeks. Prior to the experiment, mice were fed on a chlorophyll free diet (AB diets, Woerden, The Netherlands) for at least two weeks to minimize the contribution of pheophorbides to the autofluorescence emission spectrum.

Prior to the application of porphyrin precursor mice were anaesthetized using 2% Isoflurane/O_2 (Abbott, Amstelveen, The Netherlands). One group served as control receiving the vehicle alone (3% carboxymethylcellulose in water). The two other groups received either ALA or MAL cream. ALA or MAL cream (Metvix®, 160 mg MAL/g cream, Galderma, Freiburg, Germany) was topically applied to a 7 mm diameter area on the dorsal skin and covered with a thin layer of gauze. ALA cream was freshly prepared as described previously.[8] In brief, 20% (w/v) 5-aminolaevulinic acid (ALA, Medac, Hamburg, Germany) was dissolved in 3% carboxymethylcellulose in water. NaOH (2M) was added to adjust the pH to approximately 4. A polythene dressing (Tegaderm, 3M, The Netherlands) was used to occlude the cream for 4 hours. During the application period the animals were placed in a dark and warm environment. At the end of the application period the mice were again anaesthetized using 2 % Isoflurane/O_2 (Abbott, Amstelveen, The Netherlands) to remove excess cream and harvest

a 5 mm diameter punch biopsy from the centre of the area of porphyrin application. Biopsy samples were immediately frozen in liquid N_2 and stored at -80°C.

Immunohistochemical staining and 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). Vertical sections were cut and mounted on Starfrost® adhesive glass slides (Menzel, Braunschweig, Germany). Each skin sample was sectioned at 3 locations collecting 50 µm sections separated by at least 500 µm. Sections were stored at -80°C until the day before fluorescence imaging. Slides and sections were allowed to thaw at room temperature for approximately 2 hours and then carefully washed with a few drops of PBS and incubated with rat anti-mouse CD31 conjugated with Alexa Fluor® 488 (BioLegend, Uithoorn, The Netherlands) diluted 1:200 in PBS overnight at 4°C. Sections were re-washed with PBS, covered with glycerol (1:3 in PBS), sealed with nail polish and stored at 4°C prior to imaging. The spatial distribution of PpIX and endothelial cells (CD31) in the dermis up to 300 µm from the surface was imaged using a confocal microscope (Zeiss, LSM 510 Meta) in lambda mode at 400x magnification. PpIX was imaged first utilizing 405 nm excitation and spectral detection between 582 and 753 nm. Immediately thereafter Alexa Fluor® 488 was imaged under 488 nm excitation and a band pass filter (BP 530/600) or spectral detection between 493 and 753 nm. Typically 5 µm optical slices were acquired from the centre of each 50 µm section. PpIX was always imaged first in a single exposure of approximately 25s to minimize the effects of PpIX photobleaching. If too much photobleaching occured, sections were discarded and not used in the PpIX-CD31 correlation analysis. Background and reference images were recorded for each set of fluorescence images. All images were corrected for variations in excitation light intensity.

Fluorescence image analysis

A quantitative co-localisation analysis of PpIX fluorescence and CD31-AlexaFluor® 488 at 400x magnification was performed in ImageJ using software implemented by the Wright Cell Imaging Facility, according to the methods described by Li *et al.*[9] An important consideration in this type of approach is artefacts, normally termed bleed-through/overlap, caused by a significant background contribution of each fluorophore in the emission band of the other fluorophore. Preliminary experiments using control samples and singularly stained sections showed that there is no influence from PpIX fluorescence in the emission band of AlexFluor® 488 BP (530 – 600 nm) and a negligible influence of the tail of AlexFluor® 488 fluorescence

in the emission band of PpIX (620-710 nm) under 488 nm excitation. The contribution of autofluorescence in both emission bands was accounted for by performing a background subtraction based on an average uniform autofluorescence collected from control sections containing only PpIX or AlexFluor® 488 from the region of interest within which co-localisation analyses were performed.

Figure 1 shows a representative example of the co-localisation analysis. The distribution of PpIX in mouse skin after ALA and MAL application is dominated by the signal from the epidermis and stratum corneum (figure 1a) while the vasculature is confined to the dermis (7). For this reason regions of interest (ROI) are chosen to exclude the epidermis from the colocalization analysis as illustrated in Figure 1b. Figure 1c shows a single vessel in the dermis positively stained with CD31-AlexaFluor® 488. Figure 1d shows an overlay image of CD31 stained vasculature (green channel) and PpIX (red channel) in the dermis. The intensity correlation analysis[9] is based on the principle that for any set of values the sum of the differences from the mean equal zero. For two sets of random staining intensities the sum of the product of their differences will also tend to zero. If however these two intensities are dependent this product is positive and if the staining intensities are segregated the product tends to be negative. Figure 1e shows the corresponding image of this Product of the Differences from the Mean (PDM) for each pixel in the ROI where PDM = (red intensity – mean red intensity) x (green intensity – mean green intensity) using the pseudo-colour scale bar shown as shown. Pixels with a high correlation are shown in yellow and white. For each image the numbers of pixels that generate positive or negative PDM values are counted. A ratio of the number of positive values to the total number of pixel pairs is a fraction that reflects the degree of dependency. The Intensity Correlation Quotient (ICQ) is determined by subtracting 0.5 from this value where random (or mixed) staining ICQ = ~0; dependent staining 0 < ICQ <= +0.5, and for segregated staining 0 > ICQ >= -0.5. Images from multiple sections per biopsy in control animals, and in animals that received ALA and MAL (n = 5 in each) were used to determine the ICQ between the distribution of PpIX and the vasculature.

Statistical analysis

Values are presented as mean ± SD. Tests for significance between groups are performed using the normal approximation of the test ($P_{sign\ test}$) as described[9] where $p < 0.05$ was deemed significant.

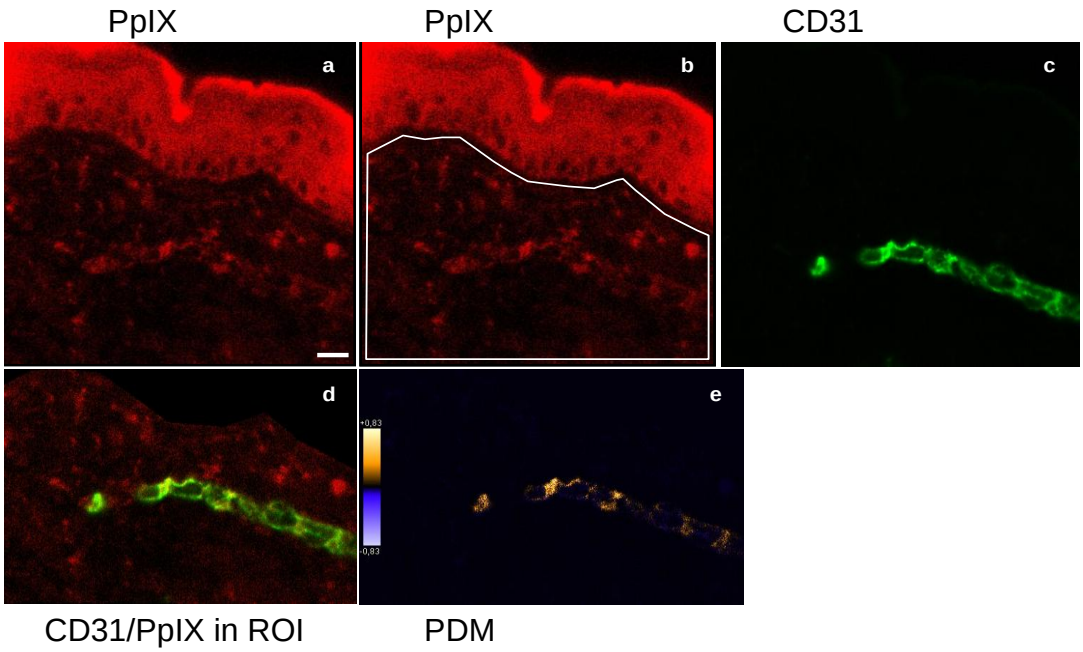

Figure 1: An example of fluorescence imaging and co-localisation analysis. Confocal PpIX fluorescence image (a) analysed over region of interest (dermis) shown in (b); CD31-AlexaFluor® 488 fluorescence image (c) and corresponding red green overlay image (d) and calculated product difference mean (PDM) image (e). The bar is 50 µm.

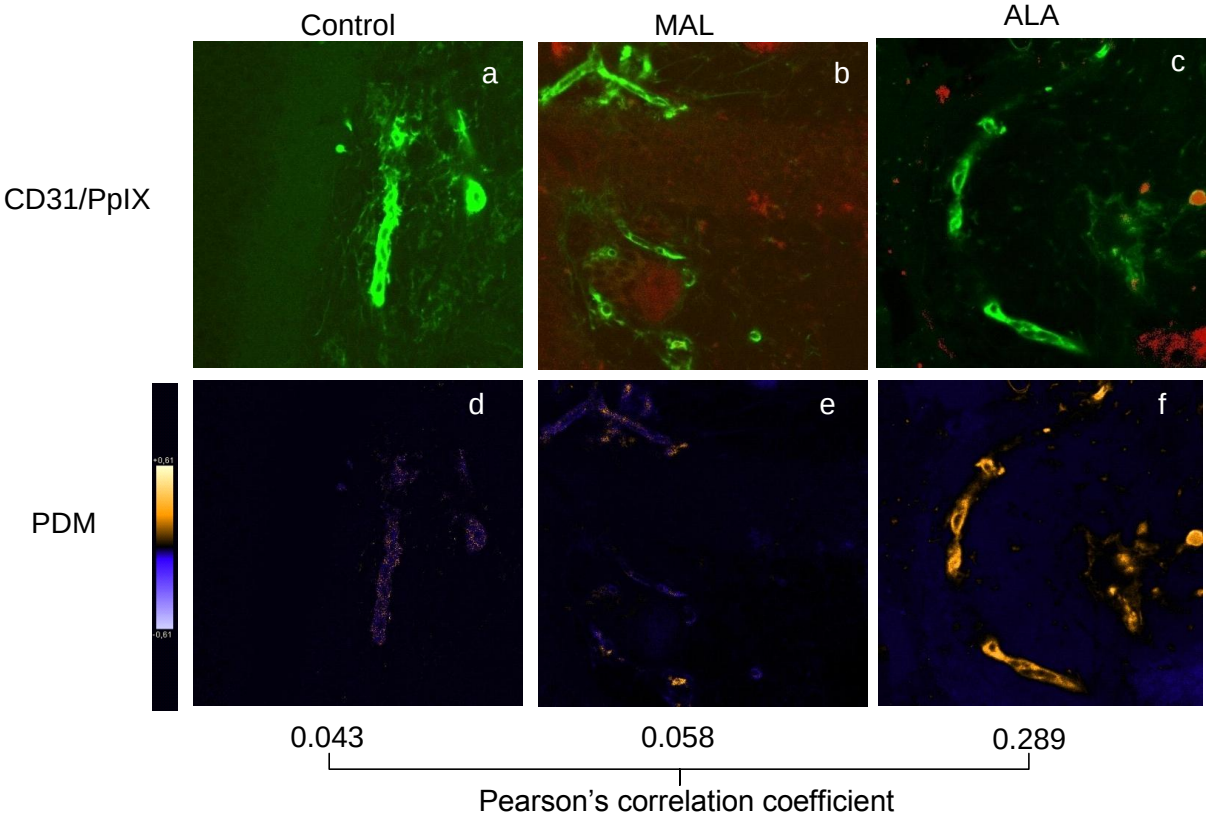

Figure 2: Dermal regions of interest from CD31-AlexaFluor® 488/PpIX overlayimages (a-c), calculated product difference mean (PDM) images (d-f) and intensity correlation quotients for representative sections from control skin and skin that has been applied with topical MAL and ALA.

RESULTS

The absence of green signal in the epidermis in figure 1c and the lack of significant red signal in the fluorescence overlay image in figure 2a illustrate no overlap in our imaging method. Figure 2 shows representative examples of PpIX, CD31-AlexaFluor®488 fluorescence images; red green overlay (a-c); and PDM images (d-f) in control animals and in animals that received MAL or ALA. The corresponding Pearson's correlation coefficient is shown below each image. There is a low positive correlation coefficient in the control image indicating a random correlation between red and green intensity. The positive correlation may be a reflection of the slight mismatch between the average background autofluorescence and the autofluorescence of the vasculature endothelium.

Four hours after the application of MAL there is a stronger correlation coefficient but there is very little PpIX fluorescence in the vasculature. The representative section after ALA application shows a much higher correlation coefficient and it is possible to visualize dependent staining between PpIX and CD31 in the overlay fluorescence image. Figure 3 shows the average data from 3 sections each in 5 animals for control biopsies and those that received MAL and ALA.

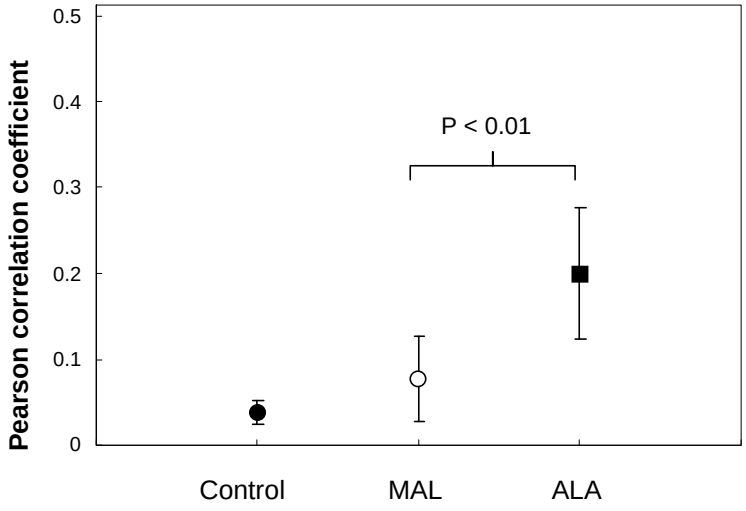

Figure 3: The average intensity correlation coefficients from 3 sections each in 5 animals for control biopsies and those that received MAL and ALA.

The average data confirm that of the representative sections shown in figure 2. There is a significantly higher correlation between PpIX and CD31-AlexaFluor® in animals that received topical ALA compared to MAL ($p_{sign} < 0.01$). There is some co-localisation of PpIX and the vasculature in some sections of skin that received MAL but on average this is not statistically significantly different from the co-localisation observed in control skin and is not greater than zero.

DISCUSSION

The aim of the present study was to investigate the distribution of PpIX after topical MAL or ALA application and to determine the degree of co-localisation of PpIX with the vasculature located in the dermis of normal mouse skin. In the epidermis, we found no significant difference in the absolute intensity of PpIX fluorescence following MAL and ALA application. However in ROIs in the dermis, within which we performed our co-localisation analysis, the distribution of PpIX shows a significantly higher degree of co-localisation with CD31 stained vasculature 4 hours after the application of ALA compared to MAL ($p<0.01$). This result is the first direct indication that the distribution of PpIX in the vasculature is different for each of these porphyrin precursors.

Before considering the implications of these results it is important to again accept that co-localisation analysis can be influenced by artefacts caused by any significant background contribution of each fluorophore in the emission band of the other fluorophore. The Stokes shift of PpIX is large enough so that there is no influence of the fluorescence emission of PpIX the detection band used for AlexFluor®488 between 530 and 600 nm. This is evidenced by the absence of fluorescence in the epidermis in figure 1c. Conversely images from control samples stained with CD31 (figure 2a) show negligible fluorescence in the emission band of PpIX from the tail of AlexFluor®488 fluorescence. Given these data we are confident that the degree of co-localisation of PpIX and CD31 that we observe is not influenced by artefacts of our methodology.

Figure 3 shows that the degree of co-localisation (ICQ) after the application of ALA (0.191±0.07) is significantly greater than that after MAL application (0.076±0.04). While the average ICQ after the application of MAL is greater than that of control tissue both are not significantly different from zero. This clearly illustrates the low localisation of PpIX in endothelial cells after the administration of MAL. The lack of a (more) complete correlation (ICQ=0.5) between CD31 staining and PpIX after the application of ALA (0.191±0.07) is probably influenced by background PpIX fluorescence outside the vasculature in the dermis caused by PpIX synthesis in cells of the dermis that are not endothelial cells e.g. (fibroblasts and mast cells).[7] Also there may be some false positive CD31 staining in other cells such as macrophages, granulocytes and neutrophils.[10]

The data of the present study supports previous findings showing that differences in the distribution of PpIX after local topical application of ALA and MAL are caused by the systemic distribution of ALA but not MAL.[11] We have now shown that as ALA undergoes local distribution into the vessels and that endothelial cells are able to synthesize PpIX. It is not known whether the concentration of MAL able to diffuse into these environments is too low to induce significant PpIX synthesis. Since we[12] and others[13] have been able to show PpIX

synthesis in endothelial cell *in-vitro* after administration of MAL, a reduced ability in endothelial cells to synthesize PpIX from MAL seems unlikely.

The absolute intensity of PpIX fluorescence in the CD31 stained vasculature after the application of ALA is difficult to compute or measure quantitatively in confocal sections but ranged from approximately 30-60% of the average fluorescence intensity at the base of the epidermis (data not shown). While the relationship between effects in the vasculature will be related to the concentration of PpIX in endothelial cells our previous findings[1] show that this level of PpIX is sufficient to generate effects such as acute oedema and inflammation, effects that are modulated by damage to the tissue vasculature.

The analysis presented in the current study did not attempt to take into account any potential influence of the depth of vessels on the extent of co-localisation of PpIX and endothelial cells in the dermis. Given the penetration profiles of ALA and MAL and the probable removal of ALA via the local vasculature in the dermis it is possible that vessels close to the surface, nearer the epidermis, would show a higher level of co-localisation between PpIX and CD31 for both ALA and MAL. To minimize this effect only the collected images were chosen so that the epidermis was in the field of view and the depth was maximal 300 µm. Given the difficulty of accurately determining the depth of individual groups of vessels in non-vertical sections of frozen tissue, this type of analysis is probably best performed using an intra-vital approach[14] by combining measuring the spatial distribution of PpIX with monitoring the response of the vasculature to therapy. It is important to note that our findings that light fractionation does not enhance the response of tissues following MAL-PDT is limited to an illumination scheme that was extensively researched and optimised for ALA. The results of the present study clearly illustrate differences between the localisation of PpIX after the application of different porphyrin precursors that seem to be related to an increase in efficacy of ALA over MAL following light fractionation. It is quite possible that the mechanism underlying this effect is related to the photosensitization of the endothelial cells that occurs following ALA-PDT. Given the widespread potential for utilising different light treatment parameters, different concentrations and formulations of other porphyrin precursors,[3,6,15] it would seem premature to discount light fractionation for porphyrin precursors other that ALA.

In conclusion, we investigated the co-localisation of PpIX and endothelial cells after topical MAL and ALA application in normal mouse skin. We found significantly higher co-localisation of PpIX and the vasculature after ALA application and low levels of PpIX in the vasculature after MAL application. These observations are in line with our previous findings that the histological response following light fractionated ALA-PDT is associated with the formation of acute oedema that is not accompanied by a different inflammatory response to PDT. This suggests that endothelial cells are involved in the difference between the response of tissue

to MAL and ALA to light fractionation and that they may be the target for enhancing efficacy using this approach.

CONFLICT OF INTEREST

The authors state no conflict of interest

REFERENCES

1. de Bruijn HS, de Haas ERM, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal mouse skin. *Photochem Photobiol Sci* 2007;**6**:1325-1331.

2. de Bruijn HS, van der Veen N, Robinson DJ, Star WM. Improvement of systemic 5-aminolevulinic acid photodynamic therapy in-vivo using light fractionation with a 75-minute interval. *Cancer Res* 1999;**59**:901-904.

3. Robinson DJ, de Bruijn HS, de Wolf J, et al. Topical 5-aminolevulinic acid-photodynamic therapy of hairless mouse skin using two-fold illumination schemes: PpIX fluorescence kinetics, photobleaching and biological effect. *Photochem Photobiol* 2000;**72**:794-802.

4. Star WM, van 't Veen AJ, Robinson DJ, et al. Topical 5-aminolevulinic acid mediated photodynamic therapy of superficial basal cell carcinoma using two light fractions with a two hour interval long-term follow-up. *Acta Derm Venereol* 2006;**86**:412-417.

5. de Haas ERM, Kruijt B, Sterenborg HJCM, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

6. Robinson DJ, de Bruijn HS, Star WM, Sterenborg HJCM. Dose and timing of the first light fraction in two fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. *Photochem Photobiol* 2003;**77**:319-323.

7. de Bruijn HS, Meijers C, van der Ploeg-van den Heuvel A, et al. Microscopic localisation of protoporphyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl 5-aminolevulinate. *J Photochem Photobiol B* 2008;**92**:91-97.

8. van der Veen N, de Bruijn HS, Berg RJ, Star WM. Kinetics and localisation of PpIX fluorescence after topical and systemic ALA application,observed in skin and skin tumours of UVB-treated mice. *Brit J Cancer* 1996;**73**:925-930.

9. Li Q, Lau A, Morris TJ, et al. A syntaxin 1, Galpha(o), and N-type calcium channel complex at a presynaptic nerve terminal: analysis by quantitative immunocolocalisation. *J Neurosci* 2004;**24**:4070-4081.

10. McKenney JK, Weiss SW, Folpe AL. CD31 expression in intratumoral macrophages: a potential diagnostic pitfall. *Am J Surg Pathol* 2001;**25**:1167-1173.

11. Moan J, Ma LW, Juzeniene AV, et al. Pharmacology of protoporphyrin IX in nude mice after application of ALA and ALA esters. *Int J Cancer* 2003;**103**:132-135.

12. Rodriguez L, de Bruijn HS, Di Venosa G, et al. Porphyrin synthesis from aminolevulinic acid esters in endothelial cells and its role in photodynamic therapy. *J Photochem Photobiol B* 2009;**96**:249-254.

13. Vallinayagam R, Schmitt F, Barge J, et al. Glycoside esters of 5-aminolevulinic acid for photodynamic therapy of cancer. *Bioconjug Chem* 2008;**19**:821-839.

14. de Bruijn HS, Kruijt B, van der Ploeg-van den Heuvel A, et al. Increase in protoporphyrin IX after 5-aminolevulinic acid based photodynamic therapy is due to local re-synthesis. *Photochem Photobiol Sci* 2007;**6**:857-864.

15. Middelburg TA, Van Zaane F, De Bruijn HS, et al. Fractionated illumination at low fluence rate photodynamic therapy in mice. *Photochem Photobiol* 2010;**86**:1140-1146.

4 Photodynamic therapy using topically applied protoporphyrin IX precursors induces vascular effects

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ABSTRACT

Light fractionated photodynamic therapy (PDT) after topical application of the porphyrin precursor 5-aminolevulinic acid (ALA) has been shown to considerably increase the efficacy compared to a single illumination. This increase in response was not observed after the application of the porphyrin-precursor methyl 5- amino- levulinate (MAL). Previous investigations have suggested that this increase may be due to the involvement of endothelial cells for ALA-PDT. Therefore in this study the differences in spatial distribution of PpIX at different depths in the skin of mice after ALA and MAL application, and the acute vascular effects of photodynamic therapy (PDT) were investigated using intra-vital confocal microscopy. ALA and MAL were topically applied for four hours. This resulted in high PpIX fluorescence intensities in the epidermis and lower, but substantial, PpIX fluorescence intensities in the dermis and subcutis for both PpIX precursors. The subcutaneous PpIX fluorescence was higher for ALA compared to MAL. This observation is remarkable since it is generally assumed that, due to poor skin penetration, PpIX synthesis induced after ALA or MAL application does not extend much further than the epidermis. The distribution of PpIX in the subcutis was inhomogeneous, with a preference for the (peri)vascular regions especially after ALA application. Subsequently, the effect of the PpIX distribution on the PDT induced vascular response was investigated. Both low (10 J/cm^2) and high (100 J/cm^2) fluence PDT induced acute vascular constriction in the dermis and subcutis. The vasoconstriction after ALA- PDT was more pronounced compared to MAL-PDT. Moreover only after ALA-PDT vascular leakage in the dermal vessels was observed, indicating endothelial damage. This study supports our previous findings that vascular responses play a role in topical PDT. Vascular damage in the dermis and more vasoconstriction for ALA in the subcutis is already induced by low fluences corresponding with the first light fraction of the fractionated illumination scheme. This supports our hypothesis that the endothelial cells are involved in the increased response of fractionated ALA-PDT.

INTRODUCTION

We recently reported on the 5 year complete response rate of superficial basal cell carcinoma following light fractionated photodynamic therapy with aminolevulinic acid (ALA-PDT).[1] We have shown that the long term response rate is significantly greater than that observed using a traditional approach utilising a single light fraction. Our interest in light fractionated ALA-PDT was raised, almost 2 decades ago, by the clinical observation that PpIX re-appeared in the time after the therapeutic illumination [W.M. Star, personal communication]. This led to a series of pre-clinical studies investigating the response of tumour and normal tissues in a number of model systems including; mammary carcinoma in skin-fold observation chambers[2] and transplanted rat rhabdoyosarcoma utilising systemically administered ALA[3] and UVB induced skin lesions[4] and normal skin following topical ALA administration.[5] Across these studies, in different model systems, light fractionation has proven particularly effective using systemic ALA administration.[2,3] Subsequent studies, aimed at optimising efficacy and elucidating the mechanism underlying the increase in response, has led to some important conclusions without providing a full understanding how they related to an increase in response *in vivo*. We, and other investigators have shown that protoporphyrin IX (PpIX) continues to be synthesised *in vivo* after PDT[6,7,8] and that cells in tissues within the treatment volume including the surrounding tumour vasculature are responsible for this re-synthesis.[9] We were confounded by the absence of a correlation between the overall amount of PpIX that is re-synthesised and the effectiveness of light fractionation[9], our original rationale for performing subsequent illuminations. We were able to show that dark intervals greater than 90 minutes led to an increase in efficacy and that a low PDT dose delivered in the first light fraction maximised efficacy. Applying the fractionated illumination scheme to PDT using the methyl ester of ALA, methyl aminolevulinate (MAL), did not lead to an increased response. Therefore our recent efforts to elucidate the mechanism underlying light fractionation have been guided by the results from two related *in-vivo* studies investigating the differences between the response following PDT with ALA and MAL. Light fractionated ALA-PDT leads to the formation of acute edema in the first few hours after treatment whereas light fractionated MAL-PDT does not.[10] Also low levels of PpIX are co-localised with cells of the vascular endothelium measured in frozen biopsies 4 hours after the topical application of ALA but not after the application of MAL.[11] Historically the vascular effects associated with topical ALA-PDT have received relatively little attention, but are now recognised as playing a potentially important role.[12] In addition to these *in-vivo* studies we have also recently reported the first *in-vitro* demonstration of the effect of light fractionation using ALA.[13] Intriguingly the efficacy of light fractionation in-vitro shows a strong dependence on the concentration of PpIX at the time of the first light fraction. Only cells incubated with

low concentrations of ALA exhibit enhanced cell death in response to light fractionated ALA-PDT. Given these insights and the small but significant concentration of PpIX in vascular endothelial cells after the topical administration of ALA, we have carefully investigated in the present study the location of PpIX *in-vivo* using intra-vital confocal microscopy and the acute vascular effects associated with light fractionated ALA-PDT.

MATERIALS AND METHODS

Outline of experimental design

The spatial distribution of PpIX and the vascular response in the skin during and after PDT was investigated using intra-vital microscopy. Two models of the skin were investigated to allow the interrogation of different tissue depths as illustrated schematically in figure 1. In the first, the epidermis and the dermis was imaged through the surface of the intact stratum corneum, in the second the subcutis was imaged from below (the side opposite to the surface of the intact skin) using a skin-fold observation chamber. Two illumination geometries were investigated, the first, that allowed high fluence PDT to be delivered, utilising a 630 nm external laser source (Zeiss, Germany) where light was directed onto the skin surface using a microlens. The second, that allowed low fluence PDT to be delivered, utilised the 514 nm output of the confocal microscope, delivered by rapid repetitive imaging of single field of view. The details of the PDT illumination parameters in these geometries are described in detail below. Window chambers were divided into three groups (table 1): Group A (n= 4) contained chambers in which the subcutis was interrogated immediately before and after high-dose PDT using a 630 nm laser under low (100x) and high (400x) magnification. Group B (n=4) contained chambers in which the distribution of fluorescence and the vasculature in the subcutis was imaged using a high magnification (400x) during PDT using low fluence 514 nm illumination. Group C (n=2) consisted of intact chambers and was used to investigate the fluorescence distribution of PpIX in the epidermis and dermis using 400x magnification during PDT using low fluence 514 nm excitation. Animals in groups B and C also received i.v. fluorescein at various time points to determine the patency of the vasculature during and after PDT.

Animal model

Dorsal skin-fold chambers were prepared on the back of 8-10 weeks old female inbred albino hairless mice (SKH1 HRCharles River, Someren, the Netherlands) under general anaesthesia (ketamine (100 mg/kg body weight) and xylazine (20 mg/kg body weight) in a mixture of 2:1:1 (v/v/v) saline, ketamine and xylazine). The method of preparation of the window chambers was slightly adapted from that described previously.[14,10] In brief, the dorsal

skin was folded and a 12 mm circular area of skin was completely removed up to the cutaneous muscular layer of opposed skin.

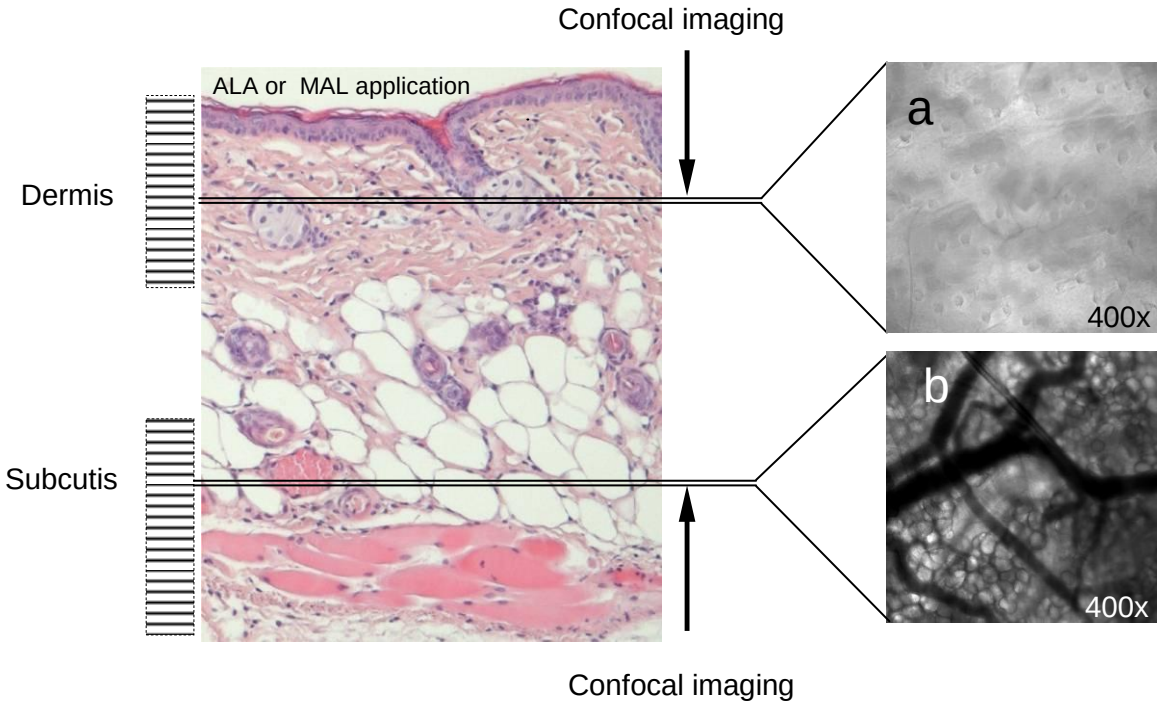

Figure 1: Schematic drawing of the two of the imaging modes of the skin to allow the interrogation of different tissue depths. In the first, the epidermis and the dermis was imaged through the surface of the intact stratum corneum, in the second the subcutis was imaged from below using a skin fold observation chamber; (a) and (b) show typical transmission images (400x magnification) in each imaging mode.

Table 1: Two models of the skin were investigated to allow the interrogation of different tissue depths

Group	Window chamber imaging subcutis	Intact skin; imaging epidermis dermis	Magnification		PDT	Imaging pre /post precursor application	Imaging pre/post PDT	Imaging during PDT
			100	400				
A (n= 4)	X		X	X	High fluence 630nm	X	X	
B (n=4)	X			X	Low fluence 514nm	X		X
C (n=2)		X		X	Low fluence 514nm	X		X

The fold was sandwiched between two frames and fixed with two light metal bolts and sutures. Spacers (9 mm circular cover slides) were used to fill the back of the chamber to position and fix the tissue close to the front. Both sides of the window were closed with a 12 mm cover slide 0.13-0.16 mm thick. To image intact skin a dorsal skin flap was folded and sandwiched between two frames and fixed with two light metal bolts and sutures. Again spacers were used to fill the back of the chamber and position the skin at the front, close to the glass. Both sides of the window were closed with a 12 mm cover slide 0.13-0.16 mm thick. Mice were housed in an ambient temperature of 28-30^0 C and humidity of 30%. Experiments started 2-4 days after operation. The committee on Animal Research of the Erasmus MC approved the experimental protocol. Mice were fed on a chlorophyll free diet (Hope Farms BV, Woerden, The Netherlands) for at least two weeks prior to the experiments to minimize the contribution of pheophorbides to the autofluorescence emission spectrum. All handlings with the mice after preparation of the chamber were performed under general anaesthesia using 2% isoflurane in oxygen (Abbott, Amstelveen, The Netherlands).

Protoporphyrin IX precursor application

Prior to the application of porphyrin precursor mice were anaesthetized. Cream was applied to the skin surface and occluded using a cover slide. Mice were allowed to regain consciousness and placed in a dark and warm environment for 4 hours after which the cream was removed and the spacers and cover slides were replaced. MAL cream was purchased from Galderma (Metvix®, 160 mg MAL/g cream, Galderma, Freiburg, Germany). ALA cream was freshly prepared as described previously.[15,11] In brief, 20% (w/v) ALA (Medac, Hamburg, Germany) was dissolved in water containing 3% carboxymethylcellulose. NaOH (2M) was added to adjust the pH to approximately 4. The control cream consisted of the vehicle alone (3% carboxymethylcellulose in water). During the application period the animals were placed in a dark and warm environment. At the end of the application period the mice were again anaesthetized to remove excess cream and to perform the imaging.

Intra-vital confocal imaging

Fluorescence and excitation light (514 nm) transmission images were recorded using a confocal intra-vital microscope (Zeiss LSM510META, Carl Zeiss B.V. Sliedrecht, The Netherlands) pre- and post cream application (all groups) and pre- and post illumination (group A) and during illuminations in groups B and C. The fields of view were carefully chosen so that they did not overlap. PpIX fluorescence was excited using the 514 nm laser and recorded in lambda mode (596-681nm, line average of 4, pixel dwell time of 6.4 microseconds, and an optical slide thickness of 40.1 µm for the 10x objective and an optical

slide thickness of 10.8 μm for the 40x objective was used). Transmission images recorded immediately after the PpIX fluorescence images using the 514 nm laser. Fluorescein fluorescence was excited using 488 nm excitation and detected using a 505-515 nm bandpass filter.

PDT illumination

All groups of mice received PDT with either a high or low fluence depending on the illumination geometry and light source. One of the goals of the present study was to investigate the dynamics of vascular response associated with the delivery of low fluence illumination. This was achieved using 514 nm excitation from the confocal microscope normally used to acquire PpIX fluorescence and transmission images of the chamber vasculature. Typically a field of 512x512 pixels (equivalent to 0.9x0.9 mm) was imaged using a pixel dwell time of 6.4 microseconds. A sequence of 8 such fields was acquired between 3 transmission images so that a total of 24 images were acquired. This resulted in approximately 12 J/cm^2 being delivered to the field of view. In this way the dynamics of PpIX fluorescence kinetics and the chamber vasculature were imaged during low fluence PDT. In order to also investigate the vascular response to high fluence PDT the skin surface of the window chamber was illuminated with a 630 nm laser (Visuals 630, Carl Zeiss B.V. Sliedrecht, The Netherlands) using a microlens to provide uniform illumination. In this configuration it was not possible to monitor changes in vascular dynamics during PDT. In order to compare the effective fluence delivered between light sources the illumination parameters at 630nm illumination were calculated to reflect typical PDT treatment parameters at 514 nm taking into account the wavelength dependent absorption of PpIX[16] and the photon energy. A fluence of 100 J/cm^2 at a fluence rate of 50 mW/cm^2 at 514 nm corresponds to a fluence of 130.6 J/cm^2 at a fluence rate of 65.3 mW/cm^2 for 630 nm light. It is important to note that while the depth dependence of penetration of light at 514 and 630 nm are significantly different, this is likely to be a small effect in a window chamber containing optically thin mouse tissue.

Vascular response measurements

During low fluence PDT FITC-BSA (Sigma) was injected i.v. at a dose of 0.2 mg/mouse immediately after illumination and images of fluorescein fluorescence were acquired between 10 and 20 minutes later to determine the acute vascular response to PDT (contraction and leakage). Similarly in groups B and C FITC-BSA was injected before low dose PDT to visualise the response of the vasculature in the skin.

Data analysis

Fluorescence images were analysed using a fit procedure, described in detail [17] written in MATLAB. The basis spectra for auto-fluorescence and PpIX fluorescence were obtained from chambers in groups (group A and B) by averaging data from 4 mice and were used to fit all fluorescence images. Regions of interest were located in the corresponding transmission images and PpIX intensity was determined in the subcutaneous skin layer, normal tissue, hair follicles, the arteriole and venule lumen and wall. Transmission images, recorded pre and post PDT (100x and 400x magnification) and during PDT (400x magnification), were used to measure the diameter and area (of the lumen) of arterioles and venules in order to determine the vascular responses. Fluorescein fluorescence images were also used to quantify the vascular response.

RESULTS

The spatial distribution of PpIX was investigated with intra-vital microscopy in mice after application of ALA, MAL and control. Two models of the skin (figure 1) were used which allowed the investigation of the distribution of PpIX and the vascular effects after PDT at different depths in the skin. Figure 2 shows a representative example of PpIX fluorescence images and corresponding transmission images 4 hours after the application of control, MAL and ALA in the subcutis for group A and B. In the transmission images arterioles en venules were clearly visible. In the control hardly any PpIX fluorescence was measured. After ALA application more PpIX fluorescence was observed compared to MAL. For both ALA and MAL the PpIX fluorescence was inhomogeneously distributed.

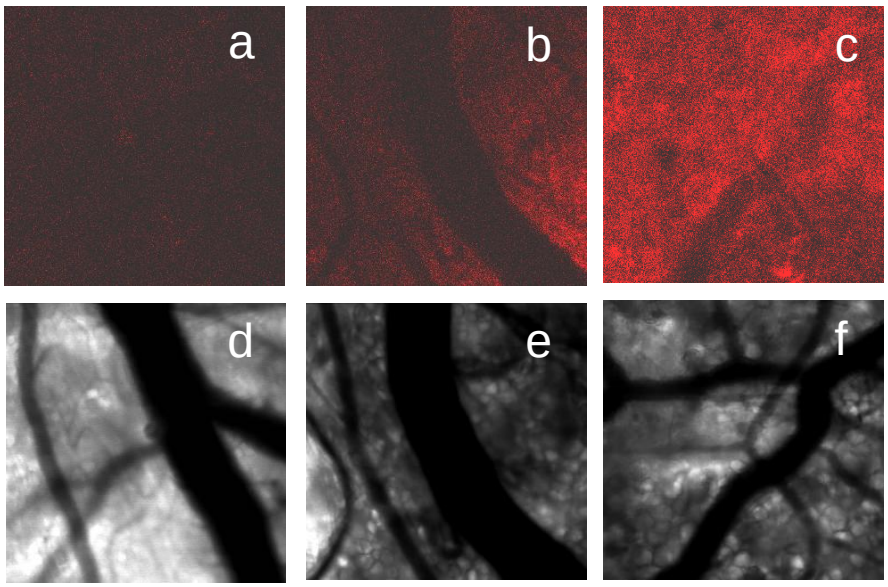

Figure 2: Representative examples of PpIX fluorescence images (a-c) and corresponding transmission images (d-f) before illumination, 4 hours after the application of control (a,d), MAL (b,e) and ALA (c,f) in animals in treatment groups A and B.

To distinguish differences in PpIX fluorescence within the subcutis the PpIX fluorescence intensities were measured in several regions of interest (a.o. adipose tissue and subcutaneous edges of the arterioles and venules). In figure 3 the mean PpIX fluorescence intensities in these regions of interest measured 4 hours after the application of control, MAL and ALA, corrected for autofluorescence, are shown. After both MAL and ALA application PpIX fluorescence is observed in the subcutis and subcutaneous vessels. More PpIX fluorescence was seen after ALA application in the arteriole walls, venule walls and adipose tissue.

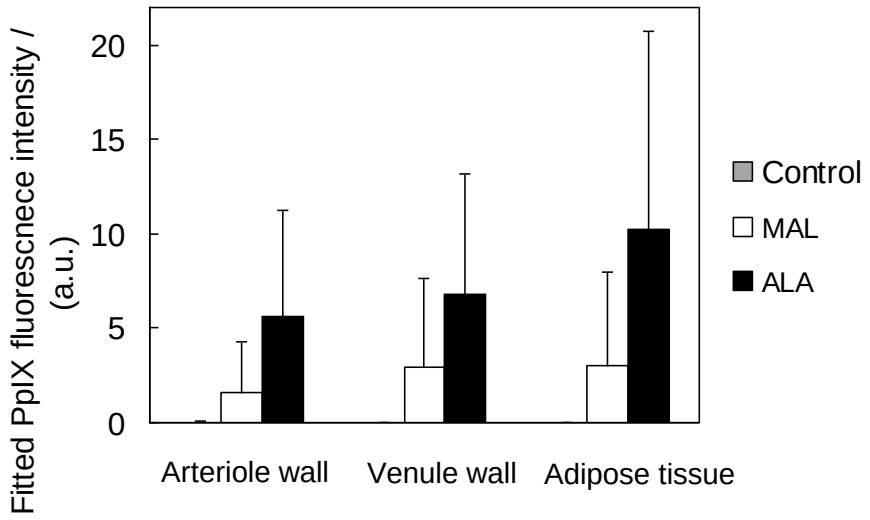

Figure 3: Mean PpIX fluorescence in regions of interest (arteriole wall, venule wall and adipose tissue) at depth in subcutis, corrected for autofluorescence, before illumination, 4 hours after the application of control, MAL and ALA in animals in treatment groups A and B (Error bars are ±SD).

The accumulation of PpIX in vessel walls 4 hours after ALA application is clearly visible in figure 4a-d. The highly fluorescent structure in a and c is a hair follicle. Relatively high PpIX fluorescence is observed in and around the arteriole (4d-f) and venule walls. In figure 4e the transmission intensities show no transmission in the vessel and some transmission around the vessels. On one side (x) of the vessel the transmission is lower and the fluorescence higher compared to the other side (y), where the transmission is relatively high. The fluorescence declines on this side, as there are no vessels present in this region of the window chamber. The fluorescence in the vessel walls originates from PpIX as shown by the spectrum in figure 4f. The effect of high dose PDT on the subcutaneous vessels was studied after application of control cream, ALA and MAL. The transmission images of arterioles and venules before and after high-dose ALA-PDT are given in figure 5.

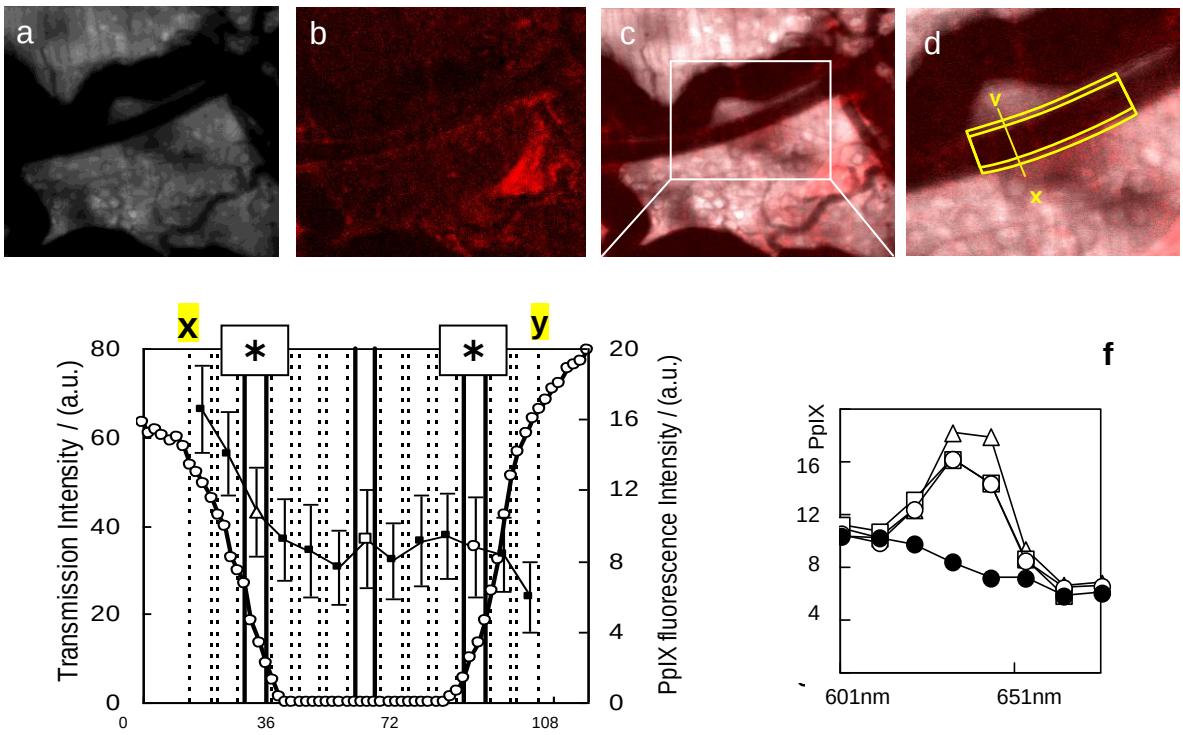

Figure 4: An overlay of PpIX fluorescence and 514 nm transmission (c) created from images (a) and transmission image (b) respectively, four hours after ALA application illustrating high PpIX fluorescence in and surrounding an arteriole and venule. Panel (d) shows an region of interest surrounding an arteriole in which longitudinal regions of interest parallel to the axis of the vessel were analysed for PpIX content. The regions of interest were 5.4 microns in diameter and approximately 120 microns long and equally spaced as indicated along the section x-y in panel d and by the vertical lines in panel e. Three locations are highlighted, the centre and each boarder of the vessel. Panel e also shows the spatial dependence of 514 nm transmitted light showing the boundary of the arteriole (*). Panel f shows the characteristic emission spectrum of PpIX in these regions of along section x-y and a corresponding average background autofluorescence signal (●) from vessels in control animals. The optical slice thickness of the fluorescence images is 40.1 microns.
(△) : x side; (☐) y side; (○) transmission.

Complete and partial arteriole contraction was seen after both ALA (5a,c) and MAL- PDT. Venule contraction was only seen after ALA-PDT in two out of four mice (5b,d) and not after MAL application. In the control group no venule and arteriole contraction was seen. In this group some dilatation after illumination was observed. In order to express the effect of high dose PDT on the subcutaneous arterioles, the average change in diameter pre- and post PDT was calculated for control, MAL and ALA (figure 6). The control group shows vasodilatation and after MAL and ALA-PDT reduction of the arteriole diameter is observed. The arteriole contraction after ALA-PDT is highest, although not statistically different from MAL-PDT. The correlation between subcutaneously arteriole diameter change and the PpIX fluorescence intensity pre-high dose PDT is given is figure 7. There is an overall correlation between PpIX intensity in the arteriole walls and change in arteriole diameter. The higher the PpIX concentration observed for ALA results in more vascular response compared to MAL.

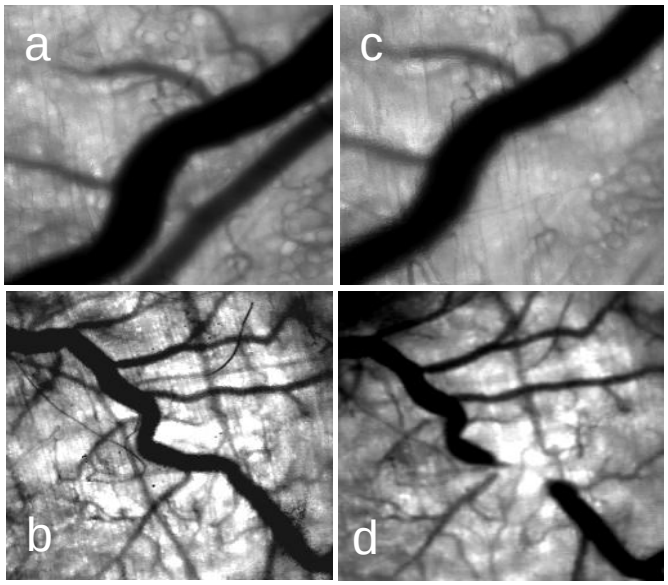

Figure 5: Transmission images of arterioles and venules pre-ALA-PDT (a, b) and post high dose ALA-PDT(c, d) (130.6 J/cm^2 at a fluence rate of 65.3 mW/cm^2 for 630nm) in group A. In image (c) complete arteriole constriction and in (d) venule constriction is visible.

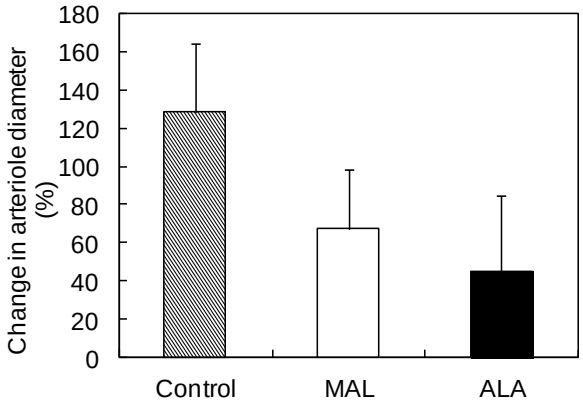

Figure 6: Change in arteriole diameter pre and post high-dose PDT(fluence of 130.6 J/cm^2 at a fluence rate of 65.3 mW/cm^2 for 630nm) for control, MAL and ALA in subcutis in group A. The control group shows vasodilatation and in MAL and ALA reduction of the arteriole diameter is observed.

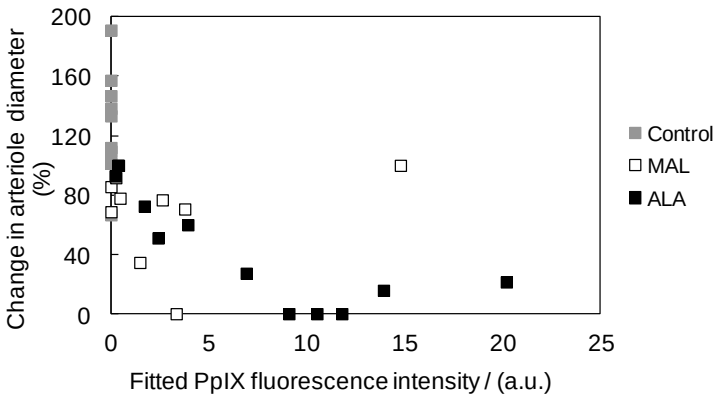

Figure 7: Correlation graph of arteriole diameter change post high-dose plotted against PpIX fluorescence intensity pre-high-dose PDT for group A.

The effect of low-dose PDT on the subcutaneous vessels (fluence of approximately 12J/cm^2 for 514nm) was studied after control, ALA and MAL application. Arteriole contraction was already observed during and after low dose ALA or MAL-PDT. More vasoconstriction was observed after and during low-dose ALA-PDT compared with MAL-PDT. An example of the difference is given in figure 8.

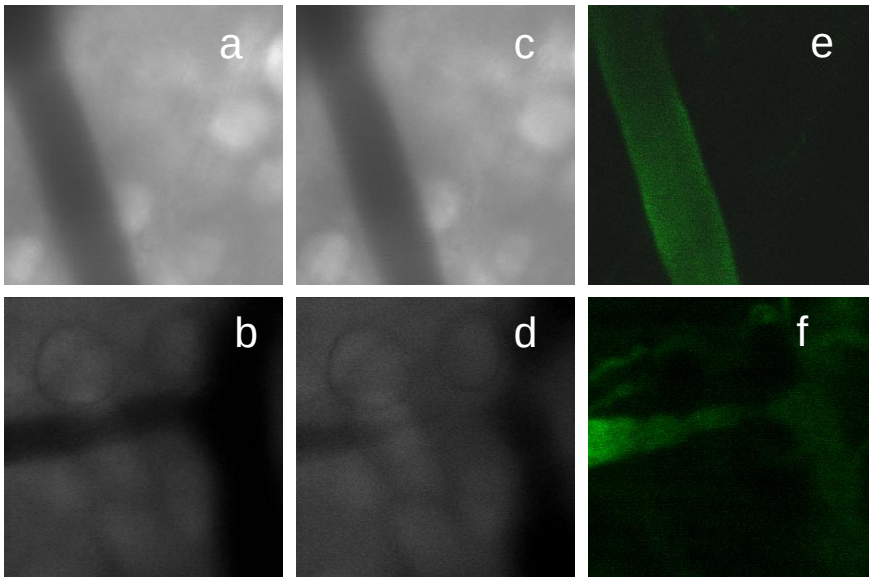

Figure 8: Transmission images pre and post low-dose-PDT (a-d) and fluoresceine fluorescence images post low-dose PDT (e,f) at 400 times magnification in the subcutis. The transmission images were obtained before and during low-dose MAL-PDT(a,c) and ALA-PDT(b,d) (fluence of approximately 12 J/cm^2 for 514nm). The fluorescein fluorescence images were acquired after low-dose MAL-PDT(e) and low-dose ALA-PDT(f). After low dose ALA-PDT partial and complete arteriole constriction is observed.

The dependence of PpIX fluorescence and vascular response for both ALA-PDT and MAL-PDT is expressed in a correlation graph of arteriole diameter change after low-dose PDT plotted against PpIX fluorescence intensity pre-low-dose PDT (figure 9). While there is a clear relationship between PpIX fluorescence intensity in the vasculature and vascular response for both ALA and MAL-PDT, there is no significant difference between ALA and MAL-PDT (p 0.76, t-test). The variation in relative arteriole area with respect to delivered fluence during low-dose PDT in vessels with the highest PpIX fluorescence intensities after MAL(a) and ALA (b) application, is shown in figure 10. Data are presented as mean, minimal and maximal response.

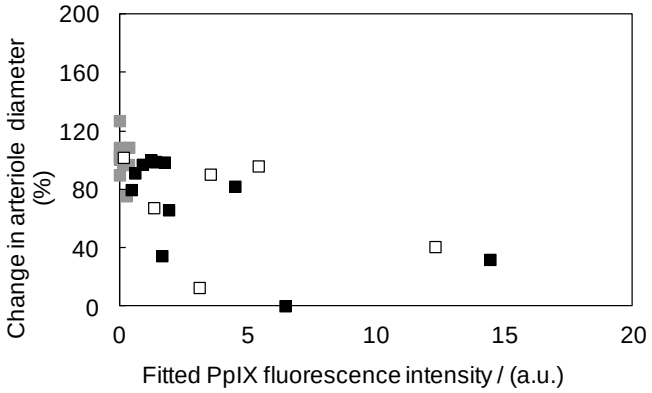

Figure 9: Correlation graph of arteriole diameter change during low-dose PDT(fluence of approximately 12 J/cm^2 for 514nm) plotted against PpIX fluorescence intensity pre low-dose PDT for control (■), MAL (□) and ALA (■) in group B.

Control animals showed no significant variation during illumination (data not shown). There is a dependence on fluence on the subcutaneous vascular response for both ALA and MAL-PDT. The dependence for ALA appears to be stronger. The effect of low dose PDT on the dermal vessels for ALA-PDT and MAL-PDT was investigated in group C by imaging through the surface of the intact stratum corneum. In figure 11 transmission images obtained after application of ALA and MAL and fluoresceine fluorescence images acquired after low-dose ALA- and MAL-PDT (fluence of approximately 12 J/cm^2 for 514nm) are shown. After low-dose MAL and ALA-PDT vasoconstriction of the capillaries is observed. Only after low-dose ALA-PDT fluorescein leakage is visible, indicating vascular leakage and damage.

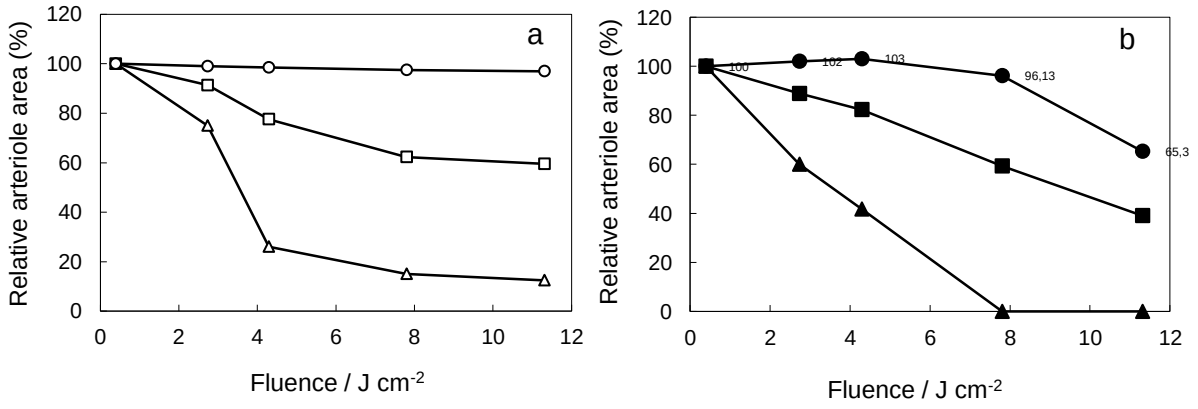

Figure 10: The variation in relative arteriole area with respect to delivered fluence during low-dose PDT, in vessels with the 4 highest PpIX fluorescence intensity (shown in figure 9) for MAL (a) and ALA (b) animals. Data are presented as mean (squares) and minimal (circles) and maximal (triangles) response. Control animals showed no significant variation during illumination (data not shown).

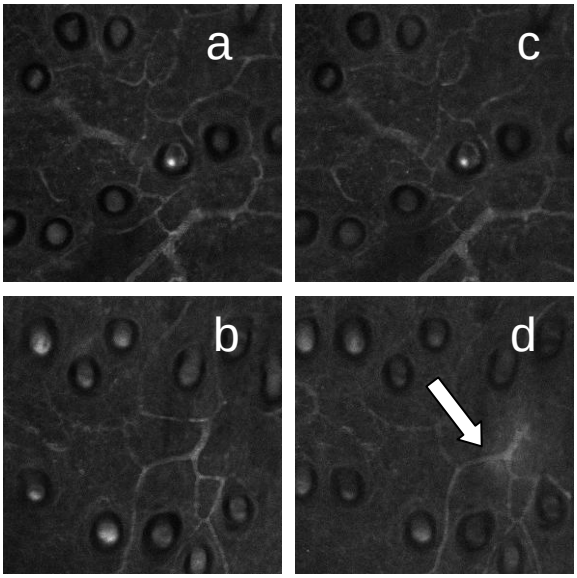

Figure 11: Transmission images (a,b) and fluorescein fluorescence images (c,d) at 400 x magnification in the dermis in group C. The transmission images were obtained before low-dose MAL-PDT (a) and ALA-PDT(b). The fluorescein fluorescence images were acquired after low dose MAL-PDT(c) and low-dose ALA-PDT(d) (fluence of approximately 12 J/cm^2 or 514 nm). After low-dose MAL and MAL-PDT vasoconstriction of the capillaries is observed. Only after low-dose ALA-PDT fluorescein leakage is visible (image d, arrow).

DISCUSSION

The aim of the present study was to investigate the spatial distribution of PpIX after the topical administration of ALA and MAL and monitoring the subsequent vascular effects during and after PDT by using confocal microscopy in an *in-vivo* model. We were unable to visualize the dermis and subcutis in one mouse model, because of too much absorption and scattering by imaging the dermis in the window chamber model from below (figure 1). Therefore we imaged the dermis through the surface of the intact stratum corneum (figure 1). Four hours after the application of ALA or MAL high PpIX fluorescence intensities were observed in the epidermis and sebaceous glands. Lower but substantial PpIX fluorescence intensities were observed in the subcutis. For both ALA and MAL the subcutaneous PpIX fluorescence was inhomogeneously distributed (figure 2).

This observation is remarkable since it is generally assumed that, due to poor skin penetration, PpIX synthesis induced after ALA or MAL application does not extend much further than the (epi)dermis[18] and that it remains restricted to the area of application, which holds especially for MAL. The PpIX concentrations in several studies are determined in *in-vitro* models or in *in-vivo* models with PpIX measurements from the surface of the skin or by extraction methods.[19,20,21] The observations on PpIX distribution described in this paper

confirm our earlier findings in vertical cryosections of excised mouse samples.[11] In this previous study high PpIX fluorescence intensities in epidermis and sebaceous glands, but also significant intensities in the dermis and subcutis were measured 4 hours after the application of ALA or MAL. After application of ALA the PpIX fluorescence intensity in the subcutis was significantly higher compared to MAL in our present and previous study.

In order to distinguish tissue regions in the subcutis responsible for the inhomogeneously distribution the PpIX fluorescence intensities were measured in several regions of interest. After ALA application more PpIX fluorescence was observed in adipose tissue and on the edges of the vessels compared to MAL (figure 3 and 4). The PpIX fluorescence observed on the outer boundaries of subcutaneous vessels originates from the endothelial cells from the arterioles, venules and the surrounding capillaries. In our model these capillaries were difficult to visualize with confocal microscopy due to the minimum optical slice thickness. However the presence of these smaller vessels could be derived through the measurement of low transmission and high PpIX fluorescence intensities in the direct surrounding of the arterioles and venules (figure 4)These observations are in accordance with our previous study in which we found significantly higher PpIX fluorescence intensities in the vessel walls after ALA application and low levels of PpIX in the vessel walls after MAL application, corrected for the perivascular PpIX concentration.[11] The lower concentrations of PpIX in adipose tissue observed after MAL application, can be explained by the more lipophilic character of MAL reducing the transfer through the epidermis. MAL gets into cells more quickly and may be suspended in higher concentration within the lipid membrane bilayer of cells in the epidermis and higher dermis compared to ALA. The more hydrophilic character of ALA may allow better penetration through the epidermis into the dermis and subcutis. This explains partly the lower PpIX concentration in the subcutaneous vessel walls after MAL application. Additional reasons for the lower PpIX concentration in the vessel walls may be the reduced uptake by the endothelial cells to induce significant PpIX synthesis due to the low perivascular MAL concentration and/or differences in uptake mechanism through the endothelial cells. The uptake of MAL by cells is different from that of ALA . ALA is taken up via active transport using carriers for β-aminoacids and GABA whereas ALA esters may be taken up by transporters other than those used for uptake of ALA or might diffuse into the cells by passive transport.[22] On the other hand indications are obtained that ALA derivatives are taken up by cells using PEPT1 or PEPT2 transporters.[23] It is unlikely that a reduced ability of endothelial cells to synthesize PpIX from MAL plays a role since we and others have been able to show PpIX synthesis in endothelial cells *in vitro.*[24,25] The effect of PpIX in the vessel walls after ALA and MAL application on the PDT-induced vascular response was investigated after high dose PDTand during low dose PDT. The high PDT dose delivered

corresponds to a therapeutic PDT dose of 100 J/cm^2 (at a fluence rate of 50 mW/cm^2 at 514 nm). This is the dose normally used for ALA-PDT or MAL PDT using a single illumination. The low fluence illumination of approximately 12 J/cm^2 illumination corresponds with the first light fraction of a two-fold illumination scheme, typically 5 - 10 J/cm^2. Both high dose and low dose PDT using topically applied ALA and MAL induces acute vascular effects in the dermis and subcutis. The vascular effects after ALA- PDT are more pronounced compared to MAL-PDT for both high dose and low dose PDT. The arteriole constriction in the subcutis after PDT is dependent on the pre-PDT PpIX fluorescence intensity in the vessel walls for both ALA and MAL. The dependence appears stronger for low and high dose ALA-PDT compared to MAL. In this study design it was not possible to investigate the vascular effects during the illumination in the high dose group. It is plausible that the vascular effect occurs early during illumination as we have shown in the low dose group a dependence of the arteriole constriction on the fluence delivered. After the delivery of fluences of 8-12 J/cm^2 a mean reduction of the relative arteriole area of 50% is observed for ALA-PDT.

The vascular effect is also provoked in the dermis after low dose PDT. After both ALA and MAL-PDT capillary/small vessel vasoconstriction is observed. Interestingly fluoresceine leakage from the capillaries in the dermis was only observed after ALA application. This leakage indicates vascular leakage and endothelial damage. PDT induces an increase in endothelial permeability, by activating RhoA, causing the breaking down of the cortical actin band to form stress fibers and then rearranging VE-cadherin, all of which leading to changes in endothelial cell morphology.[26] The thinner vessel walls in the dermis, designed for optimal exchange by diffusion of O_2 and CO_2 or transcellular transport for other molecules, are probably better permeable for ALA, resulting in higher PpIX concentration, making the small vessel walls more vulnerable than the thicker arteriole walls. This possibly explains why we did not observe fluoresceine leakage in the subcutaneous arterioles. The endothelial damage in the dermis and the vascular effects in the subcutis may explain the formation of more acute edema in the first few hours after fractionated ALA-PDT as observed in our previous study.[10] This study supports our findings that vascular responses play a role in topical ALA and MAL-PDT.[12] These results are the first indication that the vascular effect induced by topical PDT is stronger for ALA than for MAL and that this PDT vascular effect is already induced after the delivery of very low fluences. The fact that vascular damage in the dermis and more vasoconstriction in the subcutis is already induced by low fluences for ALA-PDT, corresponding with the first light fraction of a two-fold illumination scheme, supports our hypothesis that the endothelial cells are involved in the increased response of fractionated ALA-PDT.

We suggest that the low PpIX concentration observed in dermal and subcutaneous vasculature after the topical application of ALA is in the optimal range to induce vascular effects after a first low fluence light fraction. The low PpIX concentrations in the endothelial cells are of importance since de Bruijn et al. showed recently in an *in vitro*-study that the concentration of PpIX at the time of the first light fraction determined the efficacy of the therapy. Enhanced cell death in response to light fractionated ALA-PDT was only obtained in cells incubated with low concentrations of ALA. A low ALA application will result in an accumulation of PpIX still mainly located in the mitochondria. De Bruijn et al.[13] suggested that the delivery of a small PDT dose followed by a dark interval will lead to a relocalisation of PpIX making the cells more vulnerable to apoptosis or autophagy.[27,28,29] This effect was not observed after incubation with high ALA concentration, possibly overwhelming the cells leading to necrosis instead of apoptosis.[13] The second light fraction will activate PpIX, that on its turn generates singlet oxygen that will attack on mitochondrial lipids weakening the mitochondrial membrane.[30] Hereby triggering cytochrome c release and activation of the caspases cascade and apoptotic cell death. The low PpIX concentration observed after topical MAL application may be too low to induce sufficient vascular effects. In this study we concentrated on the vascular effect during the first light fraction of our twofold illumination scheme and the effects after a single high dose illumination. We did not study the effect of the second light fraction on the vascular responses.

The better understanding of the mechanism behind the increase in efficacy for fractionated ALA-PDT that we have observed in a range of models and in a long term clinical trial in the treatment of superficial basal cell carcinoma[1] may be utilized to further enhance efficacy of fractionated PDT. Most of the studies on porphyrin precursors are focused on the optimization of ALA concentration in tumor cells. However from this study it is clear that the ALA concentration in the dermal and subcutaneous vessels in and around the tumor vessels should be taken into account to optimize the response to (fractionated) PDT. Recently a liposomal formulation of ALA has been introduced, nano-emulsion BF-200 ALA, resulting in strong PpIX fluorescence signals in all layers of the epidermis. Research has been focused on the epidermal penetration in in-vitro models.[21,31] It would be interesting to investigate the liposomal ALA formulation in our window-chamber model in order to measure the PpIX concentration in the deeper layers of the skin, the co-localisation with the vasculature and the effect of low fluences. The optimization of the ALA-tissue distribution may also be used for other applications outside the skin. PDT using porphyrin pre-cursors has been applied as an experimental therapy for the treatment of several tumor types in the oral cavity, the genitourinary tract and the gastrointestinal tract and its effectiveness has been well documented.[32] A light fractionated approach, using ALA-PDT could be applied in other

organs, although it is important to note that practical and logistical barriers may be more significant than for skin. Light fractionation is not the only method that has been studied to enhance the efficacy of PDT using porphyrin precursors.[33,34,35] However modulating the delivery of light is a relatively simple practical approach that is easily achievable in a clinical setting.

REFERENCES

1. de Vijlder HC, Sterenborg HCJM, Neumann HAM, et al. Light fractionation significantly improves the response of superficial basal cell carcinoma to ALA-PDT: Five-year follow-up of a randomized, prospective trial. Acta Dermatol, 2012;**92**:641-647.

2. van der Veen N, van Leengoed HLLM, Star WM. In-vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 1994;**70**:867-872.

3. de Bruijn HS, van der Veen N, Robinson DJ, Star WM. Improvement of systemic 5-aminolevulinic acid-based photodynamic therapy in vivo using light fractionation with a 75-minute interval. *Cancer Research* 1999;**59**:901-904.

4. van der Veen N, de Bruijn HS, Berg RJW, Star WM. Kinetics and localisation of PpIX fluorescence after topical and systemic ALA application, observed in skin and skin tumours of UVB-treated mice.*Br J Cancer* 1996;**73**:925-930.

5. Robinson DJ, de Bruijn HS, de Wolf J et al. Topical 5-aminolevulinic acid-photodynamic therapy of hairless mouse skin using two-fold illumination schemes: PpIX fluorescence kinetics, photobleaching and biological effect. *Photochem Photobiol* 2000;**72**:794-802.

6. van der Veen N, de Bruijn HS, Star WM. Photobleaching during and re-appearance after photodynamic therapy of topical ALA-induced fluorescence in UVB-treated mouse skin. *Int J Cancer* 1997;**71**:110-118.

7 . Orenstein A, Kostenich G, Malik Z. The kinetics of protoporphyrin fluorescence during ALA-PDT in human malignant skin tumors. *Cancer Lett* 1997;**120**:229-234.

8. Klintenberg C af, Enejder AMK, Wang I, et al. Kinetic fluorescence studies of 5-aminolaevulinic acid-induced protoporphyrin IX accumulation in basal cell carcinomas. *J Photochem Photobiol B* 1999;**49**:120-128.

9. de Bruijn HS, Kruijt B, van der Ploeg-van den Heuvel A, et al. Increase in protoporphyrin IX after 5-aminolevulinic based photodynamic therapy is due to local resynthesis. *Photochem Photobiol Sci* 2007;**6**:857-864.

10. de Bruijn HS, de Haas ER, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated PDT in normal mouse skin. *Photochem Photobiol Sci.* 2007;**6**:1325-1331.

11. de Vijlder HC, de Bruijn HS, van der Ploeg-van den Heuvel A, et al. Differences in protopophyrin IX localization in dermal vasculature after topical application of 5-aminolevulinic acid and methyl-5 aminolevulinate. *Submitted* 2012.

12. Cottrell WJ, Paquette AD, Keymel KR, et al. Irradiance-dependent photobleaching and pain in delta-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res* 2008;**14**:4475-4483.

13. de Bruijn HS, Casas A, Di Venosa G, et al. Light fractionated ALA-PDT enhances therapeutic efficacy *in-vitro*; the influence of PpIX concentration and illumination parameters. *Photochem photobiol* 2012;Oct 29 [Epub ahead of print].

14. Seynhaeve AL, Hoving S, Schipper D, et al. Tumor necrosis factor alpha mediates homogeneous distribution of liposomes in murine melanoma that contributes to a better tumor response. *Cancer Res* 2007;**67**:9455-9462.

15. van der Veen N, de Bruijn HS, Berg RJ, Star WM. Kinetics and localisation of PpIX fluorescence after topical and systemic ALA application, observed in skin and skin tumours of UVB-treated mice. *Br J Cancer* 1996;**73**:925-930.

16. Robinson DJ, de Bruijn HS, van der Veen N, et al. Protoporphyrin IX fluorescence photobleaching during ALA-mediated photodynamic therapy of UVB-induced tumors in hairless mouse skin. *Photochem Photobiol.* 1999;**69**:61-70.

17. Kaščáková S, de Visscher S, Kruijt B, et al. In vivo quantification of photosensitizer fluorescence in the skin-fold observation chamber using dual-wavelength excitation and NIR imaging. *Lasers Med Sci* 2011;**26**:789-801.

18. Schulten R, Novak B, Schmitz B, Lübbert H. Comparison of the uptake of 5-aminolevulinic acid and its methyl ester in keratinocytes and skin. Naunyn Schmiedebergs *Arch Pharmacol* 2012;**385**:969-979.

19. Moan J, Ma L-W, Juzeniene A, et al. Pharmacology of protoporphyrin IX in nude mice after application of ALA and MAL esters. *Int J Cancer* 2003;**103**:132 – 135.

20. Forster B, Klein A, Szeimies R-M, Maisch T. Penetration enhancement of two topical 5-aminolaevulinic acid formulations for photodynamic therapy by erbium:YAG laser ablation of the stratum corneum: continuous versus fractional ablation. *Experimental Dermatol* 2010;**19**:806-812.

21. Maisch T, Santarelli F, Schreml S, et al. Fluorescence induction of protoporphyrin IX by a new 5-aminolevulinic acid nanoemulsion used for photodynamic therapy in a full-thickness *ex vivo* skin model. *Experimental Dermatol* 2009;**19**:e302-e305.

22. Rud E, Gederaas O. Hogset A, Berg K. 5-Aminolevulinic acid, but not 5-aminolevulinic acid esters is transported into adenocarcinoma cells by system beta transporters. *Photochem Photobiol* 2000;**71**:640-647.

23. Rodriguez L, Batlle A, Di Venosa G et al. Study of the mechanisms of uptake of 5-aminolevulinic acid derivatives by PEPT1 and PEPT2 transporters as a tool to improve photodynamic therapy of tumours. *Int J Biochem Cell Biol* 2006;**38**:1530-1539.

24. Rodriguez L, de Bruijn HS, Di Venosa G, et al. Porphyrin synthesis from aminolevulinic acid esters in endothelial cells and its role in photodynamic therapy. *J Photochem Photobiol B* 2009;**96**:249-254.

25. Vallinayagam R, Schmitt F, Barge J, et al. Glycoside esters of 5-aminolevulinc acid for photodynamic therapy of cancer. *Bioconjug Chem* 2008;**19**:821-839.

26. Ota H, Matsumura M, Miki N, Minamitami H. Photochemically induced increase in endothelial permeability regulated by RhoA activation. *Photochem Photobiol Sci* 2009;**8**:1401-1407.

27. Kessel D, Vicente MG, Reiners JJ. Initiation of apoptosis and autophagy by photodynamic therapy. *Lasers Surg Med* 2006;**38**:482-488.

28. Buyaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim Biophys Acta* 2007;**1776**:86–107.

29. Ji Z, Yang G, Vasovic V, et al. Subcellular localization pattern of protoporphyrin IX is an important determinant for its photodynamic efficiency of human carcinoma and normal cell lines. *J Photochem Photobiol B: Biology* 2006;**84**:213-220.

30. Kriska T, Korytowski W, Girotti AW. Role of mitochondrial cardiolipin peroxidation in apoptopic photokilling of 5-aminolevulinate-treated tumor cells. *Arch Biochem Biophys* 2005;**433**:435 446.

31. Dirschka T, Radny P, Dominicus R, et al. Photodynamic therapy with BF-200 ALA for the treatment of actinic keratosis : results of a multicentre, randomized, observer-blind phase III study in comparison with a registered methyl-5-aminolaevulinate cream and placebo. *Br J Dermatol* 2011;**166**:137-146.

32. Agostinis P, Berg K, Cengel KA, et al. Photodynamic therapy of cancer: an update. *CA Cancer J Clin.* 2011;**61**:250-281.

33. Ortel B, Chen N, Brissette J, et al. Differentiation-specific increase in ALA – induced protoporphyrin IX accumulation in primary mouse keratinocytes. *Br J Cancer* 1998;**77**:1744-1751.

34. Anand S, Honari G, Hasan T, MaytinEV. Low-dose methotrexate enhances aminolevulinate – based photodynamic therapy in skin carcinoma cells. *In vitro* and *in vivo*. *Clin Cancer R*es 2009;**15**:3333-3343.

35. Anand S, Wilson C, Hasan T, Maytin EV. Vitamin D3 enhances the apoptopic response of epithelial tumors to aminolevulinate-based photodynamic therapy. *Cancer Res* 2011;**71**:6040–6050.

5 How we perform photodynamic therapy: MAL in clinical practice

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H.A.M. Neumann

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Gold M.H. (Ed.) Springer Science, 2011:173-180.

INTRODUCTION

Photodynamic therapy (PDT) has become very popular in the last decade, especially for treating (pre)malignant cutaneous lesions. In Europe, PDT plays an important role in the everyday dermato-oncology care. Other good indications such as acne vulgaris and skin rejuvenation are not frequently treated with PDT in Europe.

Methyl-aminolevulinate (MAL) is the main photosensitizer that is used in Europe. It is commercially known as Metvix® or Metvixia® (PhotoCure ASA, Oslo, Norway) with marketing rights by Galderma (Europe/USA). Metvix® has European Union approval for the treatment of non-hyperkeratotic actinic keratoses (AKs) on the face and the scalp and for the treatment of superficial (s) and/or nodular (n) basal cell carcinoma (BCC), which are "unsuitable" for conventional therapy. Moreover, it has approval for the treatment of Bowen's disease when excision is less suitable. MAL has not yet been registered in Europe for treating other indications such as acne vulgaris and skin rejuvenation. In the USA, MAL has FDA approval for the treatment of thin and moderately thick, non-hyperkeratotic, non-pigmented AKs on the face and the scalp in immune-competent patients.

PDT is often repeated in the treatment of malignant cutaneous lesions because a single treatment yields poor long-term results. Therefore, MAL-PDT for the treatment of sBCCs and Bowen's disease, using the Galderma Metvix® protocol is repeated seven days after the first treatment. Another strategy to improve the effectiveness of PDT is by splitting the illumination scheme into two light fractions separated by a dark interval of two hours. The effect of this type of fractionation was investigated for PDT using 5-aminolevulinic acid (ALA) in preclinical and clinical studies. It was reported in the preclinical studies that the response to ALA-PDT following a two-fold illumination scheme in which the two light fractions were separated by a dark interval of two hours was significantly increased as compared with the response to a single illumination scheme.[1] Using this illumination scheme for MAL-PDT did not lead to an increased response.[4] The increase in efficacy using fractionated ALA-PDT was confirmed in randomized clinical trials.[2, 3] Differences in the biophysical and the biochemical characteristics of ALA and MAL may be important for the differences in the response to fractionated PDT. Thus, although the basic principle of MAL- and ALA-PDT may be the same, it is impossible to translate MAL to ALA and vice versa because of the difference in their chemical characteristics.

The difference in the chemical structures (Introduction Fig.4) may also be responsible for the diversity in the adverse effects such as pain during illumination. A drawback of PDT is the stinging and burning pain that can accompany treatment. The severity of pain varies from a transient discomfort to severe pain. In two clinical studies the assumption has been made that MAL-PDT produces less pain than ALA-PDT in the treatment of both acne vulgaris and non-melanoma (pre)malignant skin lesions.[5,6]

We discuss “How to use MAL” for treating non-melanoma (pre)malignant skin lesions and acne vulgaris in this chapter. The proposed treatment schemes represent “best practice” and should be updated as and when the evidence supporting the improvements becomes available.

HOW I DO IT MAL- SKIN CANCERS

There is no argument today as to whether MAL-PDT is an effective treatment for AKs and sBCCs.[7] Theoretically for carcinoma in situ (Bowen’s disease) there is also an indication for PDT, but good randomized clinical trials reporting long-term results are lacking. In our opinion, PDT is not the treatment of choice for nBCCs. Although topical MAL was reported to effectively penetrate into thick nodular BCC lesions,[8] the efficacy was lower as compared with excision.[9] Our recommendation is that (Mohs’ micrographic) surgery is the first choice of treatment for nBCCs. The following protocol for the proper use of MAL applies to Metvix® (Figure 1 a-i). Metvix® contains 160 mg/g methyl-aminolevulinate. One treatment session is recommended for the treatment of AKs. The treatment session should be repeated at seven days for the treatment of sBCCs and Bowen’s disease.

1. **Documentation:** The site, the size, the number and diagnosis of the lesion(s) should be clearly documented before starting PDT treatment. Pretreatment photographs (overview and detail) may also be useful in the accurate identification of the lesion(s). A diagnostic biopsy to establish growth pattern and thickness of the BCC needs to be considered.

2. **Lesion preparation:** Keratosis should be removed to permit optimal penetration of MAL during incubation and light during illumination. Gently remove the scales and the crusts and roughen the surface of the lesion before applying MAL. Local anesthesia is not necessary for this procedure. Pre-treatment with keratolytic agents may be considered particularly for crusted areas.

3. **Application of MAL:** MAL cream should be applied evenly in a 1 mm-thick layer on the lesion as well as on a minimum margin of 5 mm around each lesion after curettage. Many dermatologists, including ourselves have increased this margin to 10 mm. Thereafter the lesion should be occluded with an adhesive dressing (e.g. Tegaderm®, Opsite®) in order to retain the cream at site and a light protective dressing (e.g. tinfoil) to minimize the ambient light exposure. The bandages are removed and the excess cream is wiped off with 0.9% saline solution-wetted gauze after 3 hours of incubation, just before illumination.

4. **Light source and Illumination scheme:** A narrow band spectrum LED red light of 635nm (Waldmann Omnilux®) is used for illumination. A light dose of 37 J/cm^2 should be delivered with a fluence rate of 80 mW/cm^2. In principle, strictly defined doses and parameters, e.g. the distance between the light source and the lesion, are preferred to ensure that the treatment results are comparable with those in already published randomized clinical trials (RCTs). There is no need to protect the healthy skin surrounding the lesion(s) during illumination. Patients and doctor/nurse should wear suitable filter spectacles to limit the transmission of the high intensity light and to avoid discomfort and temporary disturbance in color perception during PDT. Close-fitting eye shields should be worn when the area of illumination is in the patient's face.

5. **Pain management during light illumination:** Monitor pain levels during illumination, dynamic cooling devices (desk fans) will be sufficient in most cases. Illumination may be interrupted temporarily to anesthetize the treated area if necessary. Lidocaine 1% may be administered subcutaneously (field block or nerve block). The toxic dose of 4.5 mg/kg or a total dose of 300 mg will not be reached under normal circumstances.

6. **End of treatment and aftercare:** Immediately after treatment, the treated area may appear erythematous and there may be swelling with some exudation. This will usually settle down within a day or two. Apply a non-adherent dressing. The patient may remove the dressing after 24-48 hours. The areas where MAL has been applied must be protected from sunlight for 48 hours. The treated area heals over a period of 3-6 weeks.

The treatment session (1-6) should be repeated at seven days for the treatment of sBCCs and Bowen's disease.

7. **Follow-up:** Evaluation of the response of the treated area should be performed 3 months after the treatment. If (pre)malignant lesions are visible in the treated area, then we regard this treatment failure as recurrence and will act according to the guidelines.

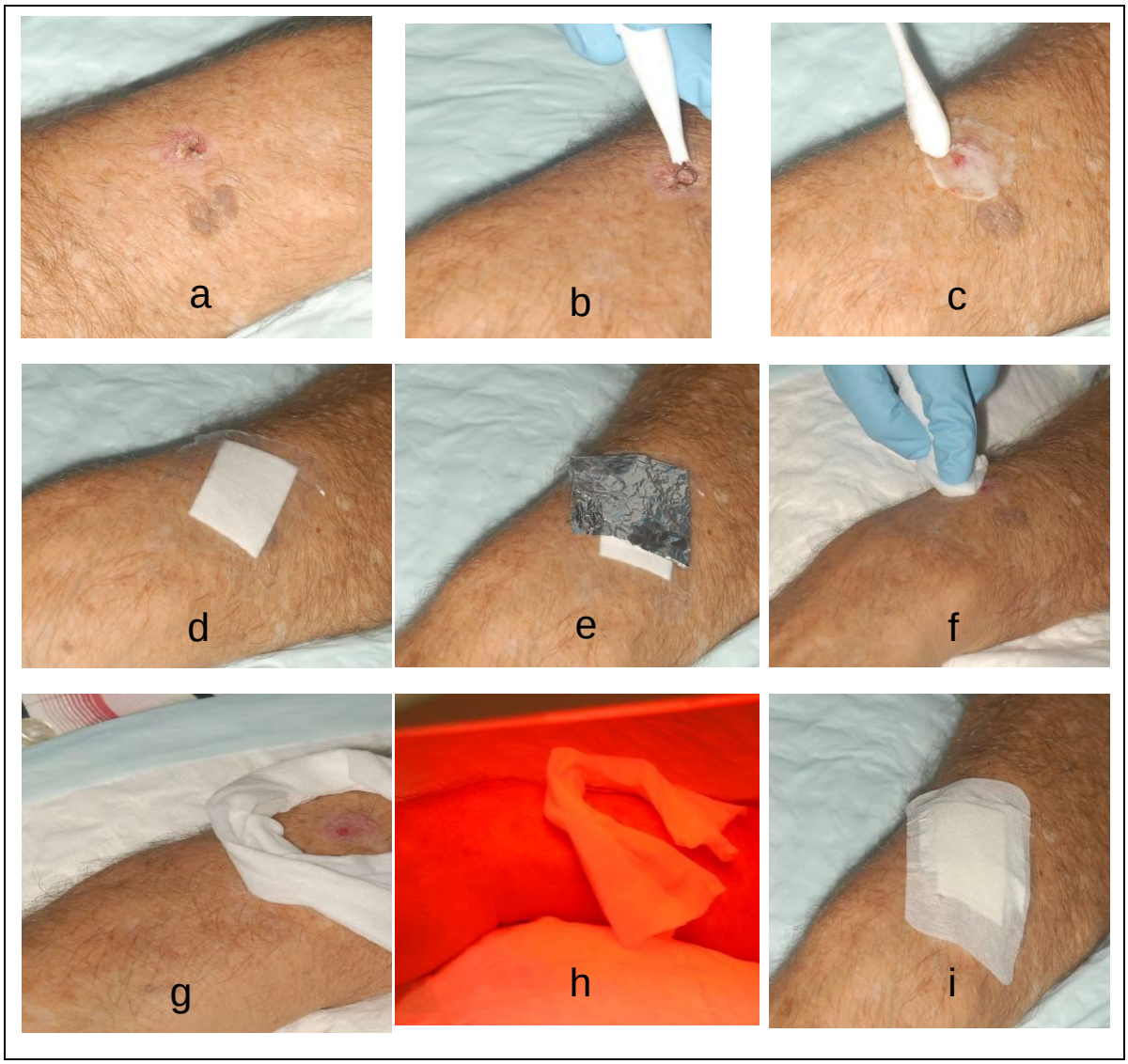

Figure 1:

a: Documentation: Pretreatment photograph of lesion (Bowen's disease).

b: Lesion preparation: Gently remove the scales and the crusts and roughen the surface of the lesion before applying MAL.

c: Application of MAL cream in a 1-mm-thick layer with a margin of 10-mm around the lesion.

d: Occlusion with an adhesive dressing (e.g. Opsite®) in order to retain the cream at the site.

e:Occlusion with a light protective dressing (e.g. tinfoil) to minimize ambient light exposure.

f:The excess cream is wiped off with a 0.9% saline solution-wetted gauze after incubation for 3 hours.

g:Lesion ready for illumination.

h: Non-coherent red light with a continuous spectrum of 570-670 nm (e.g. Waldmann Omnilux®) is used for illumination. The total light dose should be 37.5 J/cm^2 at the surface of the lesion. Therefore, the light source should be positioned at a distance that ensures even illumination of the treatment area at the desired intensity.

i: Apply a non-adherent dressing to the treated area for 24-48 hours after illumination.

Summary: How I do it MAL- Skin Cancers	
Documentation	Site, size, number, clinical diagnosis, (in case of BCC) histological examination for growth pattern, depth of lesion, pretreatment photographs.
Lesion preparation	Removal of keratoses, cream application (MAL, 160 mg/g), 10 mm border, dressings (occlusive and light-protective) for 3 hours.
Lesion treatment	Rinse off excess of cream, position light source with a defined distance (narrow band LED red light of 635 nm (Waldmann Omnilux®), light dose of 37 J/cm^2 , fluence rate of 80 mW/cm^2), monitor pain level during illumination, interrupt treatment temporarily for local anesthesia if necessary.
Lesion aftercare	Apply non-adherent dressing. Protection from sunlight for 48 hours. AKs: one treatment session, sBCCs and Bowen's disease: repeat treatment at 7 days.
Follow-up	Response evaluation at 3 months.

HOW I DO IT MAL- ACNE VULGARIS

Effectiveness of MAL-PDT was reported in several clinical trials. An improvement of acne vulgaris of 59% to 70% was reported after 1 to 4 PDT sessions using Metvix®.[5, 10-13] However, PDT was also associated with moderate to severe pain during treatment and mild to moderate adverse events such as erythema, skin swelling, pustular eruption and epithelial exfoliation after treatment were noted. Optimization of the treatment regimen is necessary for a more patient friendly approach. The majority of treatment schemes for acne vulgaris being used in the trials seems to be based on the Metvix® protocol for (pre)malignant cutaneous lesions and are presented below:

(a) Acne lesions are counted and pretreatment photographs (overview) are taken before starting PDT-treatment for comparison after the treatment.

(b) A 1-mm-thick layer of Metvix® cream is applied evenly on the face avoiding the eye area, the nose and the lips (≈ 2 grams of Metvix® for half face). The face is then occluded with a dressing for light protection. The dressing and the remaining cream are removed after 3 hours of incubation, just before illumination. The incubation time for Metvix® was 30 minutes in the study reported by Yeung et al.[13]

(c) The illumination in three RCTs was with a narrow band LED red light of 635 nm and a light dose of 37.5 J/cm^2 (Aktilite®). In one study the illumination was with non-purpuric LPDL using parameters of 7.5 J/cm^2, 10 ms, 10-mm spot size.[12] In another study the illumination was with intense pulsed light (530-750nm, 7-9 J/cm^2).[13]

(d) In the RCTs pain was managed with dynamic cooling devices or cool water sprays. All patients treated with MAL-PDT described moderate to severe pain during illumination. In at least two RCTs a few patients did not receive a second treatment because of the pain sensation during and after the first treatment.[10,11] The fluence rate was halved in one RCT in order to reduce pain during the treatment.[11] However, the effect of the halved fluence rate on pain during treatment was not compared with the fluence rate used in the other trials.

(e) After illumination the majority of patients in the RCTs experienced mild to moderate adverse events such as erythema, skin swelling, pustular eruption and epithelial exfoliation. The treatment schemes were repeated at two weeks or three weeks in the majority of the trials.

Optimization of the treatment regimen minimizing the adverse events and maintaining the efficacy is necessary before a consensus protocol for the treatment of acne vulgaris can be compiled. In a literature review by Taylor et al.[14] focused on the treatment-specific queries (the photosensitizer, the route of administration, the treatment intervals, the light sources and the patient selection) the following conclusions were drawn regarding the efficacy and the adverse events: topical short-contact (90 minutes or less) of MAL (or ALA) using a non-coherent light source at 2-4 weeks intervals for a total of two to four treatments produced the highest clinical effect. The optimum photosensitizer concentration, the wavelength of the light source and its settings are still areas needing more research. Lower MAL concentrations penetrate to shallower depths.[8] Therefore, lower concentrations may not be absorbed sufficiently to have sustained effect on the pilosebaceous units. Liposomes have the capacity to transport the encapsulated drug more selectively into the sebaceous gland.[15] A reduction in MAL concentration without losing effect on the pilosebaceous unit may be reached by encapsulating MAL in liposomes. The foundation of our choice for blue light (405nm) and a low fluence rate in the proposed treatment scheme for acne vulgaris is dealt with in more details in the discussion.

Summary: How I do it MAL- Acne vulgaris	
Documentation	Pretreatment photographs of the area to be treated.
Lesion preparation	Short-contact (90 minutes or less) application of MAL (160 mg/g), light-protective dressing during this period.
Lesion treatment	Rinse off excess cream, position non-coherent light source (blue light, 405 nm, light dose of 37 J/cm^2, fluence rate of 40 mW/cm^2).
Regimen	Treatment interval of 2-4 weeks, frequency of treatments: up to satisfaction (normally 2-4 treatment sessions).

DISCUSSION

Many clinical trials have been reported on MAL-PDT for treating non-melanoma skin cancer (NMSC) and acne vulgaris. Although response rates are high for NMSC, they do not match the response rates of (Mohs' micrographic) surgery, especially for deeper lesions. The delivery of sufficient (precursor of) PpIX and light to the full depth of the lesion and sufficient supply of oxygen are critical in topical PDT. Optimization of these three factors in order to improve the response rates for the deeper lesions is an area under investigation. Beside, pain associated with PDT is an important patient-related limitation of PDT. Pain associated with PDT is a burning, stinging pain, which usually resolves quickly but can persist for up to 24 hours and rarely up to several days.[16] The mechanisms behind the pain are not fully understood. It seems that the pain experienced during topical PDT may be directly linked to oxygen consumption and/or singlet oxygen production during light exposure.[17] The problem of pain during PDT is an issue of general concern as it may be a factor whether a treatment can be completed and/or it may be a decisive factor in a patient's choice for a next NMSC to be treated with PDT.[18] Pain has been noted to depend on several factors such as the PpIX precursor, the wavelength, the illumination settings, the protoporphyrin IX fluorescence intensity, the lesion type, size and site and patient's characteristics (e.g. age and gender). In two clinical studies the assumption has been made that MAL-PDT produces less pain than ALA-PDT in the treatment of both acne vulgaris and non-melanoma (pre)malignant skin lesions.[5,6] An explanation for the difference in pain sensation between the two porphyrin precursors may be the difference in tissue distribution.[19] The influence of the wavelength on pain was investigated recently by Mikolajewsksa et al.[17] Deeper penetrating red light (632 nm) was compared with blue light (405 nm) for ALA and MAL. They reported that ALA-PDT using red light induced pain earlier than using blue light. There was no significant difference in the pain perception between MAL-PDT using red or blue light. Based on these results Mikolajewsksa et al.[17] concluded that when deep light penetration is not necessary, the

choice of blue light for ALA-PDT is preferred. The choice of light for MAL-PDT should be determined by the area and the thickness of the lesions only, since there was no statistically significant difference between the induction times for pain with red and blue light illumination.

Low fluence rate PDT may also decrease pain during treatment. Cottrell et al.[20] did a systematic clinical investigation on the effect of irradiance on ALA-PDT efficiency and pain and they observed that lowering the irradiance reduced the pain during PDT, while maintaining the efficacy in the treatment of superficial BBCs. Wiegell et al.[21] reported that low fluence compared with high fluence MAL-PDT was associated with less pain in the treatment of acne vulgaris.

In conclusion, although a lot of research has been done into the efficiency of PDT, more work needs to be done on the effect of various treatment variables on the efficiency and adverse effects of PDT in order to develop an optimum treatment protocol for both NMSC and acne vulgaris. One should realize that the optimum treatment variables for MAL-PDT will not be automatically the optimum treatment variables for ALA-PDT and vice versa because of the differences in their biophysical and biochemical characteristics. The standardized Galderma Metvix® protocol that is used for MAL-PDT may not be the optimum protocol, but has the main advantage that treatment results are comparable with those in published randomized clinical trials.

REFERENCES

1. Robinson DJ, de Bruijn HS, Star WM et al. Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. *Photochem Photobiol* 2003;**77**:319-323.

2. De Haas ERM, Kruijt BM, Sterenborg HJ, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

3. De Haas ERM, de Vijlder HC, Sterenborg HJ, et al; Fractionated aminolevulinic acid-photodynamic therapy provides additional evidence for the use of PDT for non-melanoma skin cancer. *J Eur Acad Derm Venereol* 2008;**22**:426-430.

4. De Bruijn HS, de Haas ERM, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal mouse skin. *Photochem Photobiol Sci* 2007;**6**:1325-1331.

5. Wiegell SR, Wulf HC. Photodynamic therapy of acne vulgaris using 5-aminolevulinic acid versus methyl aminolevulinate. *J Am Acad Dermatol* 2006;**54**:647-651.

6. Moloney FJ, Collins P. Randomized, double-blind, prospective study to compare topical 5-aminolevulinic acid photodynamic therapy for extensive scalp actinic keratosis. *Br J Dermatol* 2007;**157**:87-91.

7. Braathen LR, Szeimeis R, Basset-Seguin N, et al. Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: An international consensus. *J Am Acad Dermatol* 2007;**56**:125-143.

8. Peng Q, Soler AM, Warloe T, et al. Selective distribution of porphyrins in skin thick basal cell carcinoma after topical application of methyl 5-aminolevulinate. *J Photochem Photobiol B* 2001;**62**:140-145.

9. Rhodes LE, de Rie MA, Leifsdottir R, et al. Five-year follow-up of a randomized, prospective trial of topical methyl aminolevulinate photodynamic therapy versus surgery for nodular basal cell carcinoma. *Arch Dermatol* 2007;**143**:1131-1136.

10. Horfelt C, Funk J, Frohm-Nilson M, et al. Topical methyl aminolevulinate photodynamic therapy for treatment of facial acne vulgaris of a randomized, controlled trial. *Br J Dermatol* 2006;**142**:973-978.

11. Wiegell SR, Wulf HC. Photodynamic therapy of acne vulgaris using methyl aminolaevulinate: a blinded , randomized, controlled trial. *Br J Dermatol* 2006;**154**;969-976.

12. Haedersdal M, Togsverd-Bo K, Wiegell SR, et al. Long-pulsed dye laser versus long-pulsed dye laser-assisted photodynamic therapy for acne vulgaris: A randomized controlled trial. *J Am Acad Dermatol* 2008;**58**:387-394.

13. Yeung CK, Shek SY, Bjerring P, et al. A comparative study of intense pulsed light alone and its combination with photodynamic therapy for the treatment of facial acne in Asian skin. *Lasers Surg Med* 2007;**39**:1-6.

14. Taylor MN, Gonzalez ML. Practicalities of photodynamic therapy in acne vulgaris. *Br J Dermatol* 2009;**160**:1140-1148.

15. de Leeuw J, de Vijlder HC, Bjerring P, et al. Liposomes in dermatology today. *J Eur Acad Derm Venereol* 2009;**23**:505-516.

16. Wennberg AM, Pain, pain relief and other practical issues in photodynamic therapy. *Australas J Dermatol* 2005;**46**:S3-4.

17. Mikolajewsksa, Lani V, Juzeniene A et al; Topical aminolaevulinic acid- and aminolaevulinic acid methyl ester-based photodynamic therapy with red and violet light: influence of wavelength on pain and erythema. *Br J Dermatol* 2009;**161**:1173-1179.

18. Morton CA, McKenna KE, Rhodes LE. Guidelines for topical photodynamic therapy: update. *Br J Dermatol* 2008;**159**:1245-1266.

19. Moan J, Ma LW, Juzeniene A et al. Pharmacology of protoporphyrin IX in nude mice after application of ALA and ALA esters. Int J Cancer 2003;103:132-135

20. Cottrell WJ,Paquette AD, Keymel KR, et al. Irradiance-dependent photobleaching and pain in d-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res* 2008;**14**:4475-4483.

21. Wiegell SR, Skiveren J, Philipsen PA, Wulf HA. Pain during photodynamic therapy is associated with protoporhyrin IX fluorescence and fluence rate. *Br J Dermatol* 2008;**158**:727-733.

6 Randomized comparison of photodynamic therapy with surgical excision in Bowen's disease

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ABSTRACT

Background:

Topical photodynamic therapy (PDT) using porphyrin precursors is used to treat non-melanoma skin cancers, such as Bowen's disease (BD) and basal cell carcinoma. BD is an in situ squamous cell carcinoma, with a 3-5% risk of developing into invasive squamous cell carcinoma. It occurs frequently in elderly patients and is often located at body sites associated with poor wound healing. PDT offers a non-invasive treatment of BD with the advantage of more skin preservation compared to surgical excision. Differences between the efficacy of PDT and surgery have not yet been studied in a randomized clinical trial.

Objectives:

Comparing the efficacy and cosmetic outcome of methyl aminolaevulinate (MAL)-PDT with surgical excision in the treatment of BD over minimal 18 months follow-up.

Methods:

45 patients with 48 histologically proven BD lesions from two centres were randomized to either topical MAL-PDT or excision. The MAL-PDT group was treated with 160 mg MAL/gram cream applied 3 hours before illumination to a fluence of 37 Jcm^{-2} using a narrow spectrum LED red light source (630nm) at a fluence rate of 65 $mWcm^{-2}$, repeated seven days later. Surgery was performed with an excision margin of 3 mm.

Results:

After 18 months follow-up relative complete response rate was 69.6% for MAL-PDT group and 96.0% for excision group (P=0.018, Fisher's exact test). Cosmetic outcome at 12 months was better for MAL-PDT, but not statistically significant.

Conclusions:

MAL-PDT is significantly less effective than surgical excision in the treatment of Bowen's disease, cosmetic outcome is better for MAL-PDT, but not statistically significant

INTRODUCTION

Bowen's disease (BD) is an in situ squamous cell carcinoma, that is accompanied by a 3-5% risk of developing into invasive squamous cell carcinoma.[1] It is commonly located on the lower limbs and on the head and neck and occurs more often in elderly persons with a higher risk of co-morbidities such as diabetes, atherosclerosis and/or venous insufficiency. Multiple therapeutic options are available for treatment of BD, such as surgery, cryotherapy, photodynamic therapy, 5-fluoro-uracil and imiquimod application.

Surgical excision is considered the gold standard in the treatment of BD. Relatively-low 5-year recurrence rates of 5 to 19% after surgery in non-comparative (retrospective) studies have been reported.[2] However surgery has some limitations. Cosmetic outcome, functional outcome and body sites associated with poor vascularity and healing need to be considered. For example lesions on the lower limbs of elderly patients may be complicated by poor wound healing. Therefore an effective treatment resulting in more skin preservation is desirable especially at those locations.[3] Topical photodynamic therapy (PDT) is a non-invasive therapeutic modality for the treatment of BD. PDT results in healing with little damage to normal tissue and leads to good cosmesis. PDT involves the activation of a photosensitizing agent by visible light to produce reactive oxygen species (ROS), that promote tumour destruction.[4] The topically administered prodrug of the photosensitizing agent used in dermatology, 5-aminolevulinic acid (ALA) or methyl aminolaevulinate (MAL) are converted within epidermal cells into the photosensitizer protoporphyrin IX, via the haem cycle, with preferential accumulation in tumour cells conferred by the loss of barrier function of the stratum corneum overlying the tumour cells. The efficacy for topical ALA and MAL-PDT in the treatmen of BD was shown in several open trials and randomized comparative trials. PDT provides good short term results. One year follow-up rates vary from 75% to 100%. [5,6,7,8,9,10,11,12] However after two and four year follow-up complete responses of respectively 68% and 50% are reported.[13,14] Good cosmetic outcome and higher complete response rates are achieved compared to 5-fluoro-uracil and cryotherapy.[6,7,13] However, until now the efficacy and cosmetic outcome of PDT has not been compared with surgery, the gold standard. We therefore performed a randomized, two centres, comparison study to examine the complete response and cosmetic outcome for MAL-PDT versus surgery in patients with histologically proven BD.

MATERIALS AND METHODS

Patients

Between June 2007 and July 2009, 48 BD lesions in 43 adult, otherwise healthy patients with previously untreated histologically confirmed BD, were enrolled in the open label parallel assigned randomized controlled study by AV in 2 centres, the Erasmus Medical Centre in

Rotterdam and Catherina Hospital in Eindhoven. To ensure homogeneity of the sample we excluded patients with residual and recurrence BD following other therapies, BD in the anogenital region and patients with other skin malignancies at the site of treatment, a known allergy to MAL and those that were unable to give informed consent

The study was approved by the local ethics committee responsible for each centre and conducted in accordance with the declaration of Helsinki 1975, as amended in 1996. The trial was registered in one of five ICMJE-approved public trials registries before the onset of patient enrolment (http://clinicaltrials.gov/ct2/show/record/NCT00472706). Optimal sample size (n=112 in each study arm) was determined before the start of the trial (power 80%; significance level α: 0.05) based on an expected equality between treatment groups. All patients gave written informed consent prior to entry into the study.

A blocked random allocation list was computer generated [using algorithms at randomization.com, accessed in 2006] to receive either PDT or surgery. After the inclusion of 58 histologically proven BD diameter in 52 patients, inclusion was prematurely stopped because of disappointing inclusion numbers and the disappointing clinical results for MAL-PDT. Ten lesions in 9 patients were not treated for other reasons (Diagram 1).

The patient characteristics of the 45 patients (48 lesions) across each treatment arm are given in table 1. All patients were adult Caucasians with a mean age of 71.1 years (range 54-85) and female-male ratio of 0.8 in the group receiving MAL-PDT and a mean age of 70.8 years (range 47-95) and female-male ratio of 0.7 in the group treated with excision. Most patients had 1 lesion with a maximum of two lesions. Lesion size varied from 4-30 mm in diameter, and were equally divided over the two treatment arms. In the excision treatment arm more patients were immune-compromised compared to the MAL-PDT treatment arm (8 versus 4 patients). In the MAL-PDT treatment arm more lesions were located at the extremities and less on trunk and neck (respectively 12 and 8) compared to the surgery treatment arm (respectively 8 and 12).

Table 1: Baseline characteristics of patients of both treatment arms

		MAL-PDT	Surgery
Gender Male Female		 11 9	 15 10
Age	Mean Range	71.1 (54-85)	70,8 (47-95)
Immuno-compromised	Patients Lesions	4 5	8 8
Lesions per patient	1 lesion 2 lesions	17 3	25 0
Lesion location	Trunk/neck Extremities Face/scalp	8 12 3	12 8 5

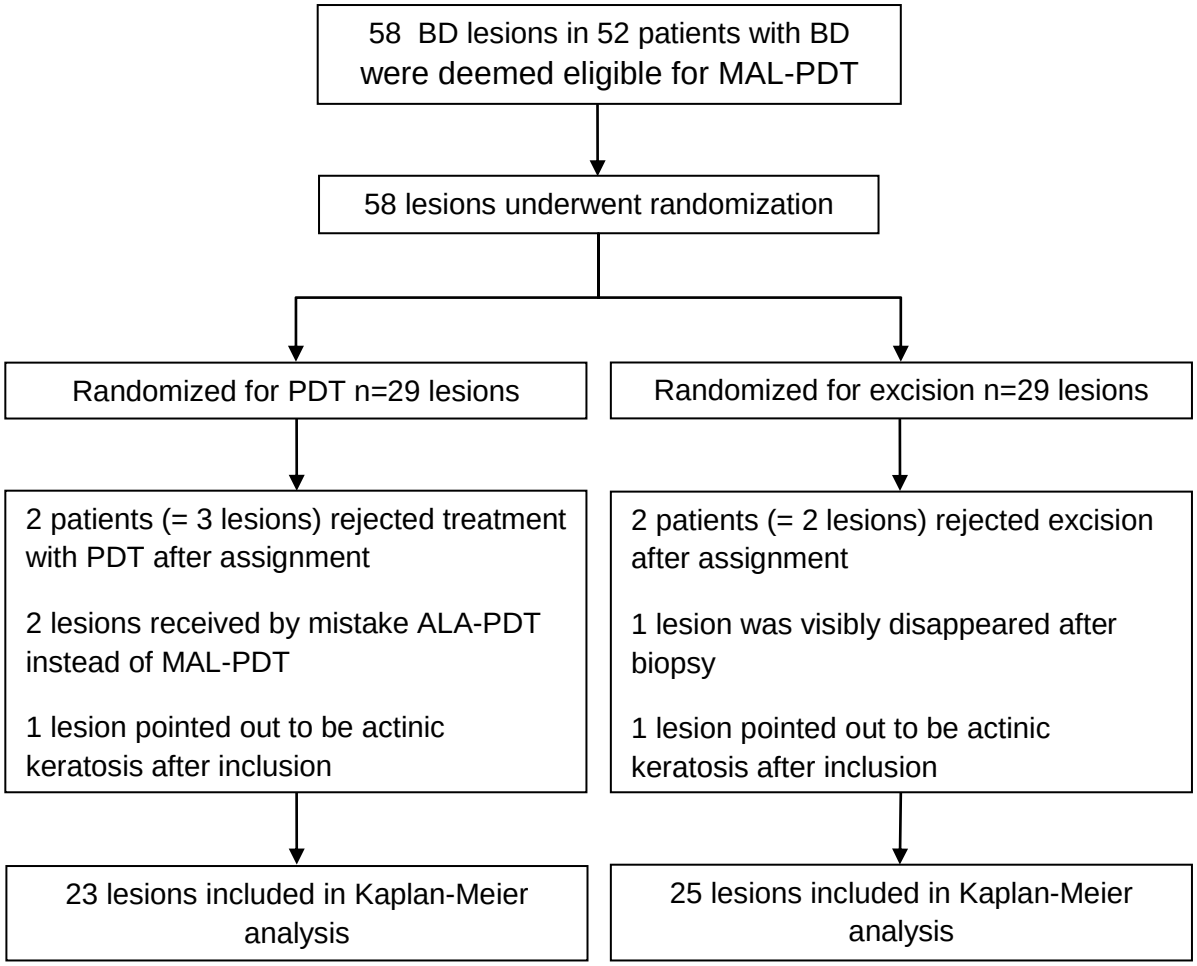

Diagram 1: Flow diagram of patient and lesion inclusion, allocation, follow-up, and data analysis of patients undergoing MAL-PDT and excision

MAL-PDT

MAL-PDT was performed, using the Galderma Metvix® protocol. MAL is commercially known as Metvix® (PhotoCure ASA, Oslo, Norway) with marketing rights by Galderma. The cream contains 160 mg MAL/g. Hyperkeratosis was removed with mild curettage before the application of MAL cream. MAL cream was applied evenly in a 1-mm-thick layer on the lesion with a minimum margin of 10 mm around the lesion and dressed with a semi-permeable dressing (Tegaderm 3M, The Netherlands) and light-protecting covering (tinfoil).

After 3 hours of incubation the excess of MAL-cream was wiped off with 0.9% saline solution-wetted gauze and the lesion was illuminated. A narrow spectrum LED red light source (Aktilite®, 630nm) was used for illumination. Desk fans were used in all 23 lesions to cool the irradiation sites during red light exposure. Lesions received 37 Jcm^{-2} at an irradiance of 65 mW cm^{-2}. Only a few patients experienced pain that required local anaesthesia (lidocaine 1% subcutaneously, field block). This treatment session was repeated at seven days.

Surgery

Lesions randomized to surgery were treated with one simple elliptical excision surgery with a 3 mm margin from the clinically estimated edge of the lesions. The excised specimen was histologically examined. Excision was performed by the staff members of the department dermatology within the two hospitals.

Outcome measures

Patients were followed up during an 18-months interval between treatment and a final follow-up assessment between December 2008 and January 2011. Follow-up was performed by our staff members and researchers within the two hospitals.

The primary outcomes were clinical response at 3, 6, 12 and 18 months. Complete response was defined as the absence of clinical visual BD lesion. Partial responses and recurrences were histologically confirmed. Recurrences, irradically excised lesions and lesions with a partial response were retreated with surgical excision and were all considered as non-responders in the statistical analysis. Subgroup analysis for lesion location (trunk/neck, extremities, face/scalp) and lesion size was performed. Lesions were stratified in two groups according to a diameter smaller than 20 mm or equal to or larger than 20 mm. Cosmetic outcome was a secondary outcome. Cosmetic outcome was assessed by patients at 12 months on a 10-point scale (0:poor, 10: excellent).

Statistics

Kaplan-Meier analysis was performed on relative complete response rates after therapy and the log-rank test was used to compare significance of differences between treatment groups. The primary and overall response rates of lesions treated with either MAL-PDT or surgery were compared using Fisher's exact test.

RESULTS

The complete responses of lesions following MAL-PDT and excision are shown is table 2 and figure 1. Eighteen months after therapy, the complete response in the excision group is 96.0%, whereas the corresponding complete response in the MAL- group is 69,6% (p= 0.018, Fisher's exact test). Of the 23 lesions in the MAL-PDT group 1 lesion failed to respond completely, 5 recurred and 1 lesion developed into squamous cell carcinoma during follow-up period (table 2). All patients completed the two PDT sessions without any serious events. The normal side effects as redness, local swelling, crust formation were seen after treatment. Of the 25 lesions in the excision group, 1 lesion was irradically excised and no lesions recurred. No wound infection or post-surgical bleeding were reported.

Table 2: Clinical response in MAL-PDT treatment arm and surgical excision treatment at 3, 6, 12 and 18 months follow-up.

Follow-up	At 3 months	At 6 months	At 12 months	At 18 months
MAL-PDT (n=23)	- 1 incomplete response - 1 SCC (2/23)	2 recurrences (4/23)	1 recurrence (5/23)	2 recurrences (7/23)
Surgery (n=25)	1 irradicaly excised (1/25)	(1/25)	(1/25)	(1/25)

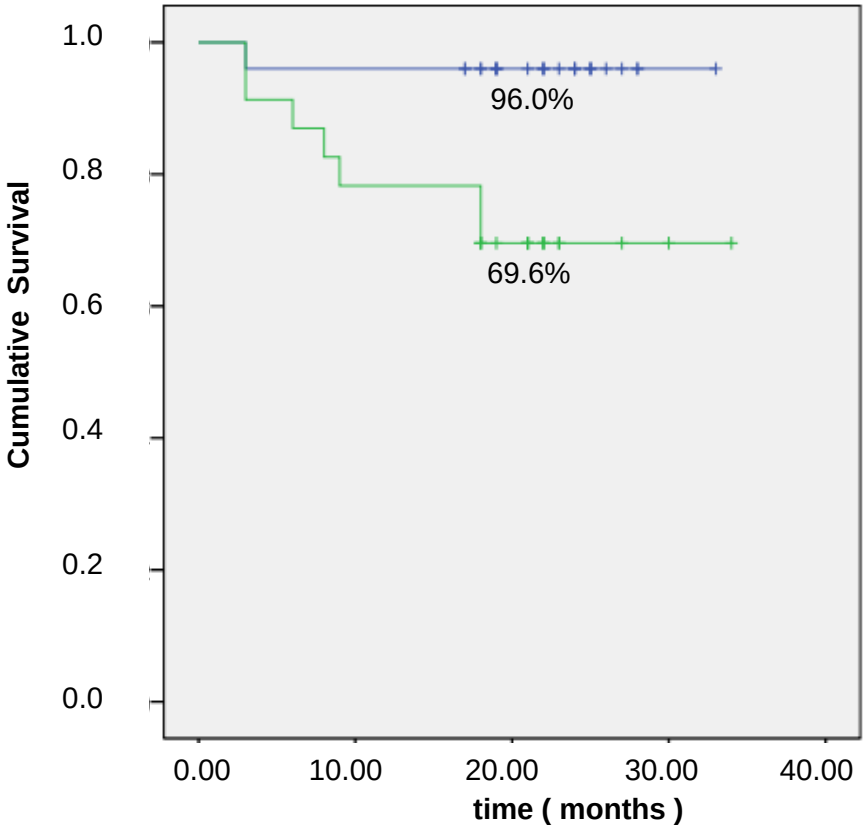

Figure 1: Kaplan-Meier curves for disease-free rates for relative complete response (CR) of BD using MAL-PDT (in green) and surgical excision (in blue) after 18months follow-up.

All non-responding lesions were retreated with surgical excision. None of the patients were lost to follow-up. One patient in the excision group died after 17 months follow-up.

A subgroup analysis for body site (trunk/neck, extremities, face/scalp) was performed for each treatment group. Lesion location did not influence the relative complete response rates in both groups.

A subgroup analysis for lesion size in the MAL-PDT group showed that relative more recurrences occurred in the group with lesion diameter equal to or larger than 20 mm compared to a diameter smaller than 20 mm. Complete responses were respectively 42.9% and 81.2%. However this difference was only border line statistically significant (p=0.06, Fisher's exact test).

Cosmetic outcome was assessed by the patients at 12 months on a 10-point scale (0: poor, 10: excellent). In the group treated with PDT the mean score was 9.0 (7-10) versus 7.9 (3-10) for surgical excision. This difference was not statistically significant.

DISCUSSION

In the treatment of BD, to our knowledge, the present study is the only published prospective randomized clinical study comparing topical PDT using porphyrin precursors and simple excision surgery, considered the gold standard treatment. BD is an *in situ* squamous cell carcinoma with an associated risk of 3-5% risk of developing into invasive squamous cell carcinoma.[1]

The results of the present study show a higher efficacy for surgical excision compared to MAL-PDT with a complete response rate of 96.0% for surgical excision and 69.6% for PDT after 18 months follow-up (p= 0.018, Fisher's exact test). Cosmetic outcome is better following PDT. Although small in patient number the differences in complete response between the treatment arms are significant. The complete response for MAL-PDT in our study is comparable with complete responses reported in open trials and randomized clinical trials regarding ALA and MAL-PDT in the treatment of BD.[5,6,7,8,9,10,11] The significant higher complete response rate for surgery is difficult to compare with literature as surgery in the treatment of BD lacks good published supportive evidence of its efficacy. Five-year recurrence rates of 5-19% after surgery and 6% after Mohs' micrographic surgery in non-comparative (retrospective) studies have been reported.[2,3,4] Our study is the only randomized clinical trial for surgery of BD and it confirms the common assumption that surgery is the treatment with highest efficacy.

This study has limitations:(**1**) All non-responding lesions in our study were retreated with surgical excision, however currently the recommended MAL-PDT regimen for BD is a repeat of the treatment cycle at 3 months if lesions are not completely cleared. Our study protocol was written and approved before the guideline on the use of PDT for NMSC was published by Braathen et al. in 2007[15], in which this regimen is recommended. Since only 1 lesion failed to respond completely at 3 months follow-up in the MAL-PDT group, the final result will not be influenced greatly (table 2). It is also important to note that most lesions recurred after the 3 months follow-up; (**2**) five of the 23 lesions in the PDT treatment arm were in immune-compromised patients, however only one of these 5 lesions recurred (at 12 month follow-up).

The main advantage of surgery compared to MAL-PDT in our study is obviously the significantly higher efficacy and the securing of histological free excision margins. However in some patient populations and body areas there may be a limitation for surgery. Analyzing the results in the MAL-PDT group shows that lesion size may be a predictive factor for recurrences. We found an association with relapse with larger diameter. This is in accordance with literature. Morton et al.[8] found in a comparative trial that the only predictor for treatment failure was the diameter (equal to or larger than 20 mm) of the BD lesion. The lesion in our study with the largest diameter in the MAL-PDT group progressed into invasive squamous cell carcinoma. While it is possible that this apparent progression may be influenced by a sampling error at the time of the diagnostic biopsy it is likely to be a manifestation of the suboptimal treatment of larger lesions, that results in residual disease. In our study lesion location did not influence the relative complete response rate which is in agreement with previous findings for MAL-PDT.[8]

The advantage of non-invasive therapies such as PDT is especially important for larger lesions at poorly vascularized locations that are associated with prolonged wound healing. It is disappointing therefore that majority of recurrences occurs in large lesions. To achieve higher complete responses, BD lesions in several trials have been treated more than once.[5,10,11] In our trial MAL-PDT was repeated at one week, according to the Galderma Metvix® protocol. Despite this approach higher efficacy in the treatment of BD has not been found.[7,8,9,10,11] A number of factors may limit the response of lesions to PDT. For example efficacy may be limited by the availability of oxygen and/or the distribution of light in tissue. The effectiveness of PDT in large lesions may be limited by the inability of the vasculature in and around the illumination area to supply oxygen for the photodynamic process. It is well documented that using a lower fluence rate, while maintaining the fluence, results in more efficient usage of the available oxygen in tissue.[16,17,18,19, 20, 21] Lowering the fluence rate of the illumination could be an interesting modification of existing MAL-PDT illumination schemes for the treatment of BD. In addition a significantly lower pain perception during PDT has been reported for low fluence PDT that may offer a significant increase in patient satisfaction.[22,23,24]

We have previously reported on the increase in complete response in the treatment of BD using ALA by altering the illumination scheme.[10] A two-fold illumination scheme in which two light fractions are separated by a substantial dark interval (2 hours) leads to a significant increase in response in various animal models[25,26,27] and in NMSC.[28,29] In the treatment of BD a complete response rate of 80% was achieved in the single illumination group at 12 months, compared to 88% in the fractionated group. Although higher, this increase did not reach statistical significance.[10] Given the potential limitations of the supply of oxygen in large lesions, the combination of fractionated ALA-PDT and low fluence rate PDT, may be an alternative approach.[30]

In conclusion, in this study we confirmed the common thought that surgery is effective in the treatment of BD. Excision has a significant higher complete response rate compared to PDT. Cosmetic outcome is better for MAL-PDT, but not significant. More (pre-)clinical research focused on an optimal illumination scheme is needed to optimize efficacy of PDT for BD.

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REFERENCES

1. Kao GF. Carcinoma arising in Bowen's disease. *Arch Dermatol* 1986;**122**:1124-1126.

2. Leibovitch I, Huigol SC, Selva DC, et al. Cutaneous squamous carcinoma in situ (Bowen's disease): treatment with Mohs micrographic surgery. *J Am Acad Dermatol* 2005;**52**:997-1002.

3. Graham JH, Helwig EB. Bowen's disease and its relationship to systemic cancer. *Arch Dermatol* 1961;**83**:738-758.

4. Thestrup-Pedersen K, Ravnborg L, Reymann F. Morbus Bowen. A description of the disease in 617 patients. *Acta derm venereol* 1988;**68**:236-239.

5. Ball SB, Dawber RPR. Treatment of cutaneous Bowen's disease with particular emphasis on the problem of lower leg lesions. *Australian J Dermatol* 1998;**39**:63-70.

6. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol* 1992;**55**;145-157.

7. Morton CA, Whitehurst C, Moore JV, MacKie RM. Comparison of red and green light in the treatment of Bowen's disease by photodynamic therapy. *Br J Dermatol*, 2000;**143**:767-772.

8. Morton CA, Whitehurst C, Moseley H, et al. Comparison of photodynamic therapy with cryotherapy in the treatment of Bowen's disease. *Br J Dermatol* 1996;**135**:766-771.

9. Salim A, Lehman JA, McColl JH, et al. Randomized comparison of photodynamic therapy with topical 5-fluorouracil in Bowen's disease. *Br J Dermatol* 2003;**148**:539-543.

10. de Haas ERM, Sterenborg JJCM, Neumann HAM, et al. Response of Bowen's disease to ALA-PDT using a single and 2-fold illumination scheme. *Arch Dermatol* 2007;**143**:264-265.

11. Wong TW, Sheu HM, Lee JY. Photodynamic therapy for Bowen's disease (squamous cell carcinoma in situ) of the digit. *Dermatol Surg* 2001;**27**:452-456.

12. Calzavara-Pinton PG, Venturini M, Sala R et al. Methylaminolaevulinate-based photodynamic therapy of Bowen's disease and squamous cell carcinoma. *Br J Dermatol* 2008;**159**:137-144.

13. Morton CA, Horn M, Lehman JA, et al. A 24-month update of a placebo controlled European study comparing MAL-PDT with cryotherapy and 5-fluorouracil in patients with Bowen's disease. *J Eur Acad Derm Venereol* 2005;**19**:237-238.

14. Dragieva G, Hafner J, Dummer R et al. Topical photodynamic therapy in the treatment of actinic keratoses and Bowen's disease in transplant recipients. *Transplantation* 2004;**77**:115- 121.

15. Braathen LS, Szeimies RM, Basset-Sequin N, et al. Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: An international consensus. *J Am Acad Dermatol* 2007;**56**:125-143.

16. Langmack K, Mehta R, Twyman P, Norris P. Topical photodynamic therapy at low fluence rates-theory and practice. *J Photochem Photobiol B* 2001;**60**:37-43.

17. Finlay JC, Conover DL, Hull EL, Foster TH. Porphyrin bleaching and PDT-induced spectral changes are irradiance dependent in ALA-sensitized normal rat skin in vivo. *Photochem Photobiol* 2001;**73**:54-63.

18. Robinson DJ, de Bruijn HS, van der Veen N, et al. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. *Photochem Photobiol* 1998;**67**:140-149.

19. Foster TH, Murant RS, Bryant RG, et al. Oxygen consumption and diffusion effects in photodynamic therapy. *Radiat Res* 1991;**126**:296-303.

20. Sitnik TM, Henderson BW. The effect of fluence rate on tumor and normal tissue responses to photodynamic therapy. *Photochem Photobiol* 1998;**67**:462-466.

21. Henderson BW, Busch TM, Vaughan LA, et al. Photofrin photodynamic therapy can significantly deplete or preserve oxygenation in human basal cell carcinomas during treatment, depending on fluence rate. *Cancer Res* 2000;**60**:525-529.

22. Clark C, Bryden A, Dawe R, et al. Topical 5-aminolaevulinic acid photodynamic therapy for cutaneous lesions: outcome and comparison of light sources. *Photodermatol Photoimmunol Photomed* 2003;**19**:134-141.

23. Cottrell WJ, Paquette AD, Keymel KR, et al. Irradiance-dependent photobleaching and pain in delta-aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res* 2008;**14**:4475-4483.

24. Wiegell SR, Haedersdal M, Philipsen PA, et al. Continuous activation of PpIX by daylight is as effective as and less painful than conventional photodynamic therapy for actinic keratoses; a randomized, controlled, single-blinded study. *Br J Dermatol* 2008;**158**:740-746.

25. de Bruijn HS, van der Ploeg-van den Heuvel A, Sterenborg HJ, Robinson DJ. Fractionated illumination after topical application of 5-aminolevulinic acid on normal skin of hairless mice: the influence of the dark interval. *J Photochem Photobiol B* 2006;**85**:184-190.

26. Robinson DJ, de Bruijn HS, Star WM, Sterenborg HJ. Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. *Photochem Photobiol* 2003;**77**:319-323.

27. van der Veen N, van Leengoed HL, Star WM. In vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 1994;**70**:867-872.

28. de Haas ER, Kruijt B, Sterenborg HJ, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

29. de Vijlder HC, Sterenborg HCJM, Neumann HAM, et al. Light fractionation significantly improves the response of superficial basal cell carcinoma to ALA-PDT: Five-year follow-up of a randomized, prospective trial, *Acta Dermatol* 2012;**92**:641-647

30 . Middelburg TA, Van Zaane F, De Bruijn HS, et al. Fractionated illumination at low fluence rate photodynamic therapy in mice. *Photochem Photobiol* 2010;**86**:1140-1146.

7 Oculocutaneous albinism and skin cancer risk

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ABSTRACT

Background: Oculocutaneous Albinism(OCA) comprises a group of genetic disorders characterized by a diffuse dilution of pigmentation within melanocytes and keratinocytes of the skin, the hair follicles and the eyes. Differences in the sensitivity to skin carcinogenesis may be expected depending on the type of mutation in oculocutaneous albinism. The assertion that the risk for developing skin cancer in albinos is most evident in patients without any pigmentation can be challenged.

Objective: It is known that the presence of pheomelanin and precursors cause an increase in the concentration of reactive oxygen species (ROS). Therefore, it is expected that the risk for developing skin cancer in albinos with low amounts of pheomelanin will be higher than that in albinos without any pigmentation.

Methods: A literature search was performed in order to obtain a better insight into the relationship between various types of albinism and the risk of skin cancer.

Results and conclusion: Although it is known that albinos have a large risk to develop skin cancer, the current literature fails to provide convincing evidence for the differences in the risks between the various types of albinism. Since pheomelanin induces ROS, which renders especially red-haired people more susceptible to skin cancer, we conclude that albinos having pheomelanin in the keratinocytes must be more susceptible to skin cancer than albinos without any melanin. An indication that this may be the case is that the white skin of those with vitiligo, devoid of melanocytes and thus unable to produce any melanin, is resistant to melanomas, basal cell- and squamous cell carcinomas.

INTRODUCTION

There are large differences in the susceptibility between various skin types to develop skin cancer upon exposure to sunlight. When DNA is damaged, p53 oncogene mutations are observed in more than 90% of all skin cancer cases.[1]

The nuclear genome of the keratinocytes is protected against the ultraviolet light by melanosomes located mainly above the nuclei of the keratinocytes. This type of photo-protection reduces the penetration of light and quenches the formation of ROS and prevents DNA damage. The protection of the skin for UV radiation is strongly dependent on the degree of pigmentation for which various types of melanin polymers are responsible. These polymers are classified into three groups: DHI-eumelanin, insoluble brown to black pigments, lighter coloured DHICA-eumelanin and reddish-brown pheomelanin (Fig.1).[2]

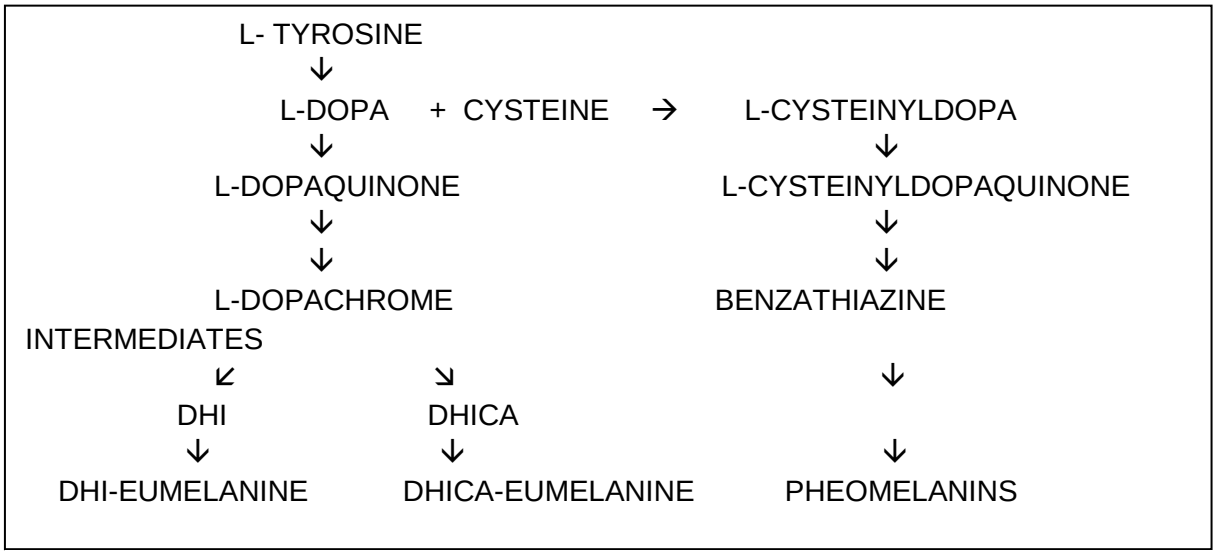

Figure 1*:* Major steps in melanogenesis. DHI: 5, 6-dihydroxyindole, DHICA: 5, 6-dihydroxyindole carboxylic acid.

OCULOCUTANEOUS ALBINISM (OCA)

Individuals with mutations in melanosomal enzymes that lead to melanin deficiency, the so-called albinos, are highly susceptible to UV-induced skin cancer.

Mutations disrupting tyrosinase, tyrosine-related-protein-1, P-protein or MATP activity are known to induce oculocutaneous albinism (OCA) (Table 1). TYR gene mutations disrupting tyrosinase activity result in the disturbance of the melanin polymer synthesis. Both OCA1A and OCA1B are caused by more than 200 mutations in the TYR gene known to be associated with albinism. In OCA1A, tyrosinase is completely inactive and melanin synthesis is inhibited throughout the patient's life. Therefore, the phenotype of OCA1A is characterized by completely white hair and skin and red pupils. This is in contrast to OCA1B in which tyrosinase activity is decreased. Patients with OCA1B develop yellow pigment in hair, eyes and skin throughout life. OCA1 has a prevalence of about 1 per 40,000. It is very uncommon

among African-Americans.[3] TYR gene mutations disrupting tyrosinase activity result in the disturbance of the melanin polymer synthesis. Both OCA1A and OCA1B are caused by more than 200 mutations in the TYR gene known to be associated with albinism. In OCA1A, tyrosinase is completely inactive and melanin synthesis is inhibited throughout the patient's life. Therefore, the phenotype of OCA1A is characterized by completely white hair and skin and red pupils. This is in contrast to OCA1B in which tyrosinase activity is decreased. Patients with OCA1B develop yellow pigment in hair, eyes and skin throughout life. OCA1 has a prevalence of about 1 per 40,000. It is very uncommon among African-Americans.[4] OCA2 is caused by mutations in the P-protein. The P-protein is important for normal processing, transport and activity of melanosomal proteins regulating melanosomal pH.[5] Patients with OCA2 may appear at birth to have OCA1A, but throughout life they develop a phenotype that resembles OCA1B. OCA2 is the most common type of albinism in Black Africans.(Fig 2) The prevalence of OCA2 is estimated to be 1 in 36,000 in the USA, but is 1 in 3,900 in South-Africa.[6] Mutations in tyrosine-related protein-1 can lead to OCA3.[7] OCA3 is a common type of albinism in southern African blacks. Clinical features of OCA3 are rather mild, with red hair and reddish-brown skin. This mild phenotype prevents detection of OCA3 in Caucasian and Asian populations.[8]Mutations in the SLC24A5, encoding for MATP, a potassium dependent sodium-calcium exchanger,[9] cause OCA4. More than 30 different mutations have been found in various populations, but there is a high prevalence in Japan.[10] The clinical phenotype of OCA4 is variable and is similar to OCA2.

Table 1: Types of OCA and the affected genes, gene products and phenotype

Types of OCA	**Gene**	**Affected protein**	**Phenotype**
OCA1A (OMIM 203100)	TYR	Tyrosinase absent/inactive	white hair, pinkish skin, red pupils
OCA1B (OMIM 606952)	TYR	Tyrosinase partially active	yellow pigment in hair, eyes and skin
OCA2 (OMIM 203200)	OCA2	P-protein	similar to OCA1B
OCA3 (OMIM 203290)	TYRP1	Tyrosinase-related protein-1	red hair, reddish-brown skin
OCA4 (OMIM 606574)	SCL45A2	MATP	similar to OCA1B & 2

UV-induced skin cancer

UV radiation is the main factor that affects pigmentation and is a risk factor for developing various types of skin cancer. Chromophores that are hit by UVB radiation are generally aromatic and heterocyclic ring structures (pyrimidines, purines, aromatic amino acids and melanin). Pyrimidine dimers formed after UVB radiation is described as UVB signature.[11] Modified chromophores can transfer their energy leading to ROS[12], acting as second messengers in activating signalling cascades.[13] However, they also cause damage to DNA, lipids and proteins. DNA damage is normally repaired by the nucleotide excision repair system.

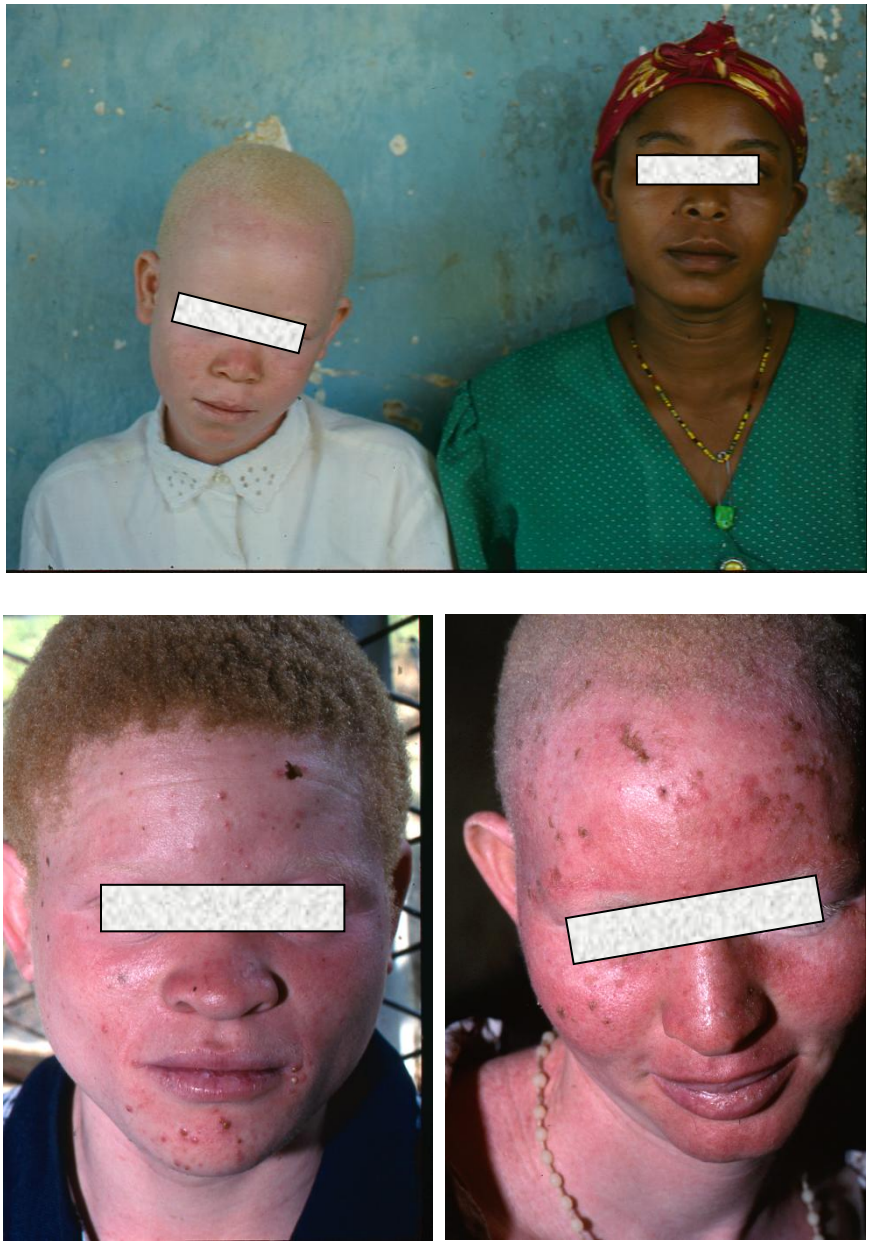

Figure 2: Typical examples of African child with phenotype of OCA1B or OCA2.
(Photographs taken by J. Van der Stek, Erasmus MC, Rotterdam, The Netherlands)

The cells are arrested in the G1 or G2 phase of the cell cycle regulated by p53 for overhauling the damage. In undamaged cells, p53 is ubiquitous and degraded, resulting in low concentrations. DNA damage increases p53 concentrations arresting the cell cycle.Accumulation of UV-related damage leads to increased ROS and P53 mutations so that cell division is no longer restrained resulting in skin cancer.[14] Melanin polymers protect the nuclear genome against UV-induced damage, but pheomelanin also causes an increase in ROS.

DISCUSSION

It is generally accepted that OCA, caused by a failure to synthesize melanin, is an established risk factor for developing UV-induced skin cancer.[15,16] Kromberg et al[15] suggested that the presence of some pigment in OCA(1B, 2, 3 and 4) patients offering some photoprotection will decrease skin cancer risk. The assumption that a low level of pigment reduces the risk of developing skin cancer, can be challenged. We suggest that OCA1A patients will have a lower risk of developing skin cancer as compared with the other OCA types. This is supported by the fact that the melanin polymer pheomelanin is mainly produced when melanogenesis remains at a basal level as that in OCA1B, 2, 3 and 4. Pheomelanin promotes the production of ROS. The damaging effect of ROS on DNA is well known.[17] This is clearly seen in patients with xeroderma pigmentosum (an autosomal recessive genetic disorder of DNA repair), with a 2000-fold increase in the incidence of skin cancer after exposure to UV radiation. This leads to multiple (metastases of) squamous cell carcinoma or melanomas[18] and even death.[19] As OCA1A patients do not synthesize melanin the risk to produce UV-induced ROS is lower. Another argument that OCA1A patients may be less vulnerable for skin carcinoma comes from the comparison with vitiligo. In patients whose white spots have no melanin, a negative correlation between vitiligo and skin cancer has been reported.[20] An explanation of this may be that increased levels of glutathione peroxidase and superoxide dismutase (SOD) inactivate ROS, thus providing protection against oxidative damage and thereby reducing the risk of skin cancer.[21] It would be worthwhile to examine this in the white skin of OCA1A patients. To distinguish between the susceptibilities to UV-induced skin cancer the various mutations causing OCA should be considered together with studies on single nucleotide polymorphisms (SNPs) showing significant association with skin cancer risk as identified in genome-wide association studies (GWAS) or replication studies [22]

In conclusion, we state that the absence of pheomelanins and possible ROS inactivation by anti-oxidative enzymes make that the risk of UV-induced skin cancer in OCA1A patients may be less than in the other OCA types. To obtain a valid comparison of the risk of skin cancer between OCA1A and the other types of albinism, research to the difference in ROS

concentration must be performed. Also the information obtained from SNPs, associated to skin cancer risk, must be taken into account possibly providing further insights into the molecular mechanism(s) of skin cancer.

REFERENCES

1. Rodust PM, Stockfleth E, Ulrich C et al. UV-induced squamous cell carcinoma- a role for antiapoptotic signalling pathways. *Br J Dermatol* 2009;**161**:107-115.

2. Parra E.J. Human Pigmentation Variation: Evolution, Genetic Basis, and Implications for Public Health. *Yearbook Phys Anthropol* 2007;**50**:85-105

3. Grønskov K, Ek J, Brondum-Nielsen K. Oculocutaneous albinism (review) *Orphanet J Rare Diseases* 2007;**2**:43 doi:10.1186/1750-1172-2-43.

4. Grønskov K, Ek J, Brondum-Nielsen K. Oculocutaneous albinism (review) *Orphanet J Rare Diseases* 2007;**2**:43 doi:10.1186/1750-1172-2-43.

5. Puri N, Gardner JM, Brilliant MH. Aberrant pH of melanosomes in Pink-Eyed dilution (p) mutant melanocytes. *J Invest Dermatol* 2000;**115**:607-613.

6. Kromberg JG, Jenkins T. Prevalence of albinism in the South African negro. S Afr Med J 1982;**61**:383-386.

7. Toyofuku K, Wada I, Valencia JC, et al. Oculocutaneous albinism types 1 and 3 are ER retention diseases: mutation of tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins. *FASEB J.* 2001;**15**:2149-2161.

8. Tomita Y, Suzuki R. Genetics of Pigmentary Disorders. *Am J Med Gen* 2004;131C:75-81.

9. Fukamachi S, Shimada A, Shima A. Mutations in the gene encoding, a novel transporter protein, reduce melanin content in medaka. *Nat Genet* 2001;**28**:381-385.

10. Suzuki T, Tomita Y. Recent advances in genetic analyses of oculocutaneous albinism types 2 and 4. *J Dermatol Sci* 2008;**51**:1-9.

11. Nishigori C. Cellular aspects of photocarcinogenesis. *Photochem Photobiol Sci* 2006;**5**:208-214.

12. Scandalios JG. The rise of ROS. *Trends in Biochem Sci* 2002;**27**:483-486.

13. Van Laethem A, Garmyn M, Agostinis P. Starting and propagating apoptotic signals in UVB irradiated keratinocytes. *Photochem Photobiol Sci* 2009;**8**:299-308.

14. Tilly CMLJ, Van Steensel MAM, Krekels GAM, et al. Molecular aetiology and pathogenesis of basal cell carcinoma (a review). *Br J Dermatol* 2005;**152**:1108-1124.

15. Kromberg JG, Castle D, Zwane EM, Jenkins T. Albinism and skin cancer in Southern Africa. *Clin Genet* 1989;**36**:43-52.

16. Asuquo ME, Otei OO, Omotoso J, Bassey EE. Skin cancer in albinos at the University of Calabar Teaching Hospital, Calabar, Nigeria. *Dermatology on Line* 2010;16.

17. Rees JL. The genetics of sun sensitivity in humans. *Am J Hum Genet* 2004;**75**:739–751.

18. Cleaver JE, Lam E, Revet L. Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. *Nat Rev Genet* 2009;**10**:756-768.

19. English JSC, Swerdlow AJ. The risk of malignant melanoma, internal malignancy and mortality in xeroderma pigmentosum patients. *Br J Dermatol* 1987;**117**:457-461.

20. Schallreuter KU, Tobin DJ, Panske A. Decreased photodamage and low incidence of non-melanoma skin cancer in 136 sun exposed Caucasian patients with vitiligo. *Dermatology* 2002;**204**:194-201

21. Feily A, Pazyar N. Why vitiligo is associated with fewer risk of skin cancer?: Providing a molecular mechanism. *Arch Dermatol Res* 2011;**303**:623-624.

22. Gerstenblith MR, Shi J, Landi MT. Genome-wide association studies of pigmentation and skin cancer: a review and meta-analysis. *Pigment Cell Melanoma Res* 2010;**23**:587-606.

8 From clinical study to daily practice: A translational turn still to be made

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ABSTRACT

Optimization of treatment modalities is highly important in reducing the demands on the public health care system. Translational research offers the possibility to implement such a reduction. This type of research may be divided into T1, in which the knowledge of the disease mechanism is converted into new methods for the diagnosis, the therapy and/or the prevention - the 'bench to bedside' component, and T2 component in which clinical studies are translated into the daily practice. Skin cancer, particularly non-melanoma skin cancer, is reaching epidemiological proportions in the Netherlands imposing an ever increasing care burden on an increasing number of physicians. Various gains and pitfalls of translational medicine via translational research into optimization of photodynamic therapy used in the treatment of non-melanoma skin cancer are elucidated in this communication.

INTRODUCTION

Translational medicine is research aimed at translating basic scientific research to patient care. The emphasis lies on the coupling between laboratory and the patient, also referred to as "from bench to bedside". However, the actual implementation in the general health care, whereby health gains may be realized for the whole population is underexposed, but certainly not less important. In 2008, Woolf attempted to define both these phases of translational research: translation 1 (T1) and translation 2 (T2).[1] T1 was described as the transfer of new knowledge of disease mechanisms discovered in the laboratory to the development of new methods for the diagnosis, the therapy and/or the prevention in addition to the first clinical studies in people. T2 was defined as the translation of the results of clinical studies to the daily practice and the influence of that on the most important decision moments in the process of diagnosis to treatment (health decision making). A model with the various steps in translational research is presented in Figure 1.[2] In this model the constant exchange of knowledge, dialogue and interaction between researchers and users is important to achieve the ultimately intended improvement in the health care. It is important to examine whether implementation of the new methods in the general health care actually provides health gains for the whole population and which factors play a role therein.

Translational medicine is important for achieving maximum health gains for the whole population for disorders such as skin cancer which has a high incidence in the society. Skin cancer continues to reach epidemiological proportions in the Netherlands. The incidence of skin cancer has steadily increased in the past decade. More than 1 in 6 Dutch individuals suffers from some form of skin cancer, particularly non-melanoma skin cancer, before the age of 85 years.[3]

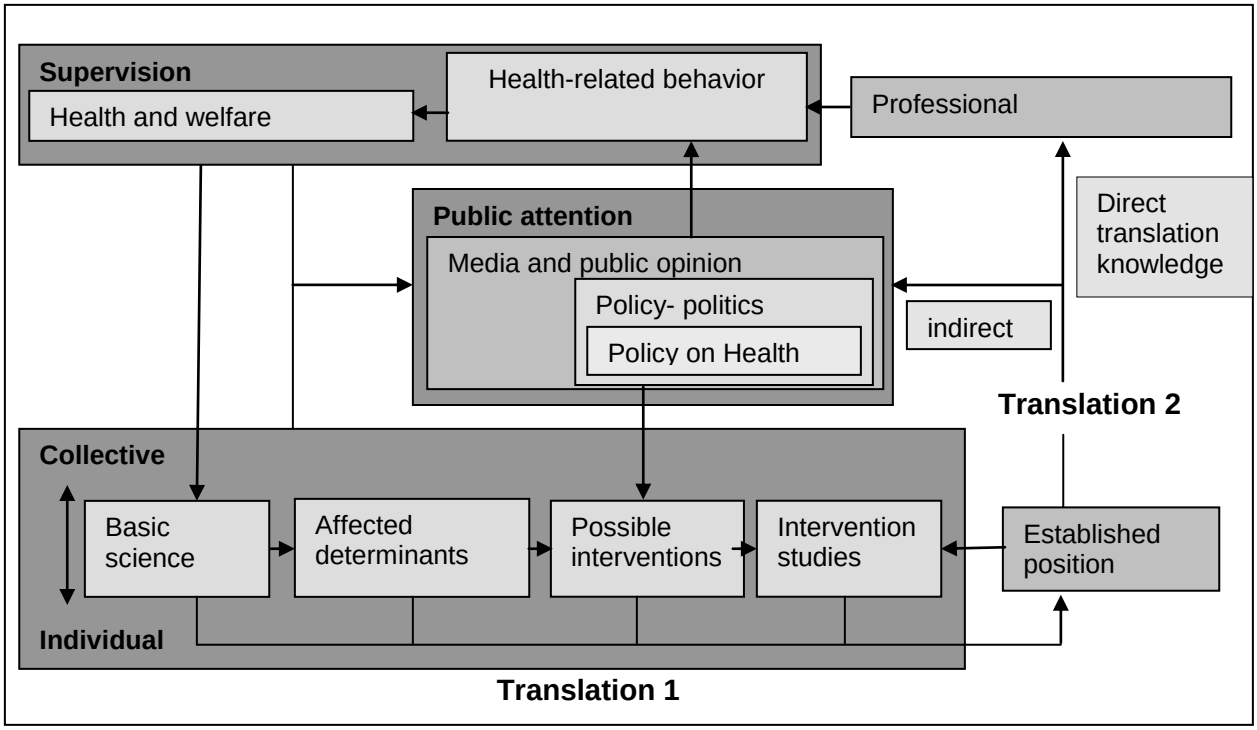

Figure 1: Model with the various steps in translational research based on Olgilvie et al, 2009.[2] In this model a continuous exchange of knowledge, dialogue and interaction between investigators and users is important in order to achieve the intended ultimate improvement in the health care.

The burden of skin cancer is mainly disease burden, functional and cosmetic problems and care consumption because of the good prognosis (quod vitam). General practitioners and medical specialists will be increasingly burdened with the inspection, diagnosing and/or treatment of suspect lesions. Optimization of the treatment modalities is highly important in order to reduce the burden on the health care system because preventive measures have had no effect thus far and the care capacity for early detection is too low.

In this article, various gains and pitfalls of translational medicine via translational research into optimization of photodynamic therapy (PDT) used in the treatment of non-melanoma skin cancer at the Erasmus MC are elucidated. The working mechanism of PDT is briefly dealt with before that.

Mechanism of PDT

Topical PDT is used in the treatment of superficial non-melanoma (pre)malignancies such as actinic keratosis, Bowen' disease (intraepithelial carcinoma) and superficial basal cell carcinoma. The mechanism is based on the activation of a photosensitive agent by visible light of a suitable wavelength in the presence of oxygen. The ideal photosensitive agent selectively accumulates in the target cells. The photosensitive agent is excited during exposure to light followed by

reactions with cellular material and the formation of reactive oxygen. This results in cell death and thus destruction of the target tissue.

Two protoporphyrin IX (PpIX) precursors: 5-Aminolaevulinic acid (ALA) and methyl-aminolaevulinate (MAL) are used in dermatology. Conversion into PpIX and subsequently into haem occurs after topical application and up-take of the precursors in the keratinocytes. The synthesis of PpIX is regulated by a feedback mechanism in which haem inhibits the synthesis of ALA. The negative feedback is circumvented by exogenous administration of ALA or MAL whereby there is an intracellular accumulation of PpIX, particularly in the (pre)malignant keratinocytes. Hereby, the (pre)malignant cells are mainly destroyed during the exposure to light. ALA is a hydrophilic molecule. Moreover, it is a zwitterion at physiological pH, bearing both a positive charge at the amino group and a negative charge at the carboxylic acid group. Such compounds are assumed to have limited capacity to cross biological barriers such as cell membranes and the stratum corneum. The more lipophilic methyl ester of ALA, MAL, was introduced to get a deeper penetration. MAL is registered in Europa under the name Metvix®. Despite the higher lipophilicity, elevated intracellular PpIX concentrations after MAL administration in comparison with ALA haven't been shown *in vitro 4* and in *vivo.*5The Metvix® supplier advises to repeat the MAL-PDT a week after the first treatment in order to guarantee adequate effectiveness. In the Netherlands, Metvix®-PDT is used more than ALA-PDT. In contrast, ALA-PDT is used more often in North America. A difference in the effectiveness between MAL-PDT and ALA-PDT has not been demonstrated. An extensive description of the topical PDT was reported by Thissen et al.6

Translation 1

Fundamental research with the aim of developing new methods and optimizing the already existing methods is imperative considering the ever increasing incidence of skin cancer. Therefore, attempts were made to increase the effectiveness of topical PDT by esterification of ALA to MAL in the past years.

Preclinical studies into the effect of various light illumination parameters in ALA-PDT were pursued in the 1990s at the Erasmus MC. It was discovered that the effectiveness was improved by fractionated illumination instead of a single illumination.7 This finding formed the foundation of the first translation of our research (see overview in Figure 2).8,9

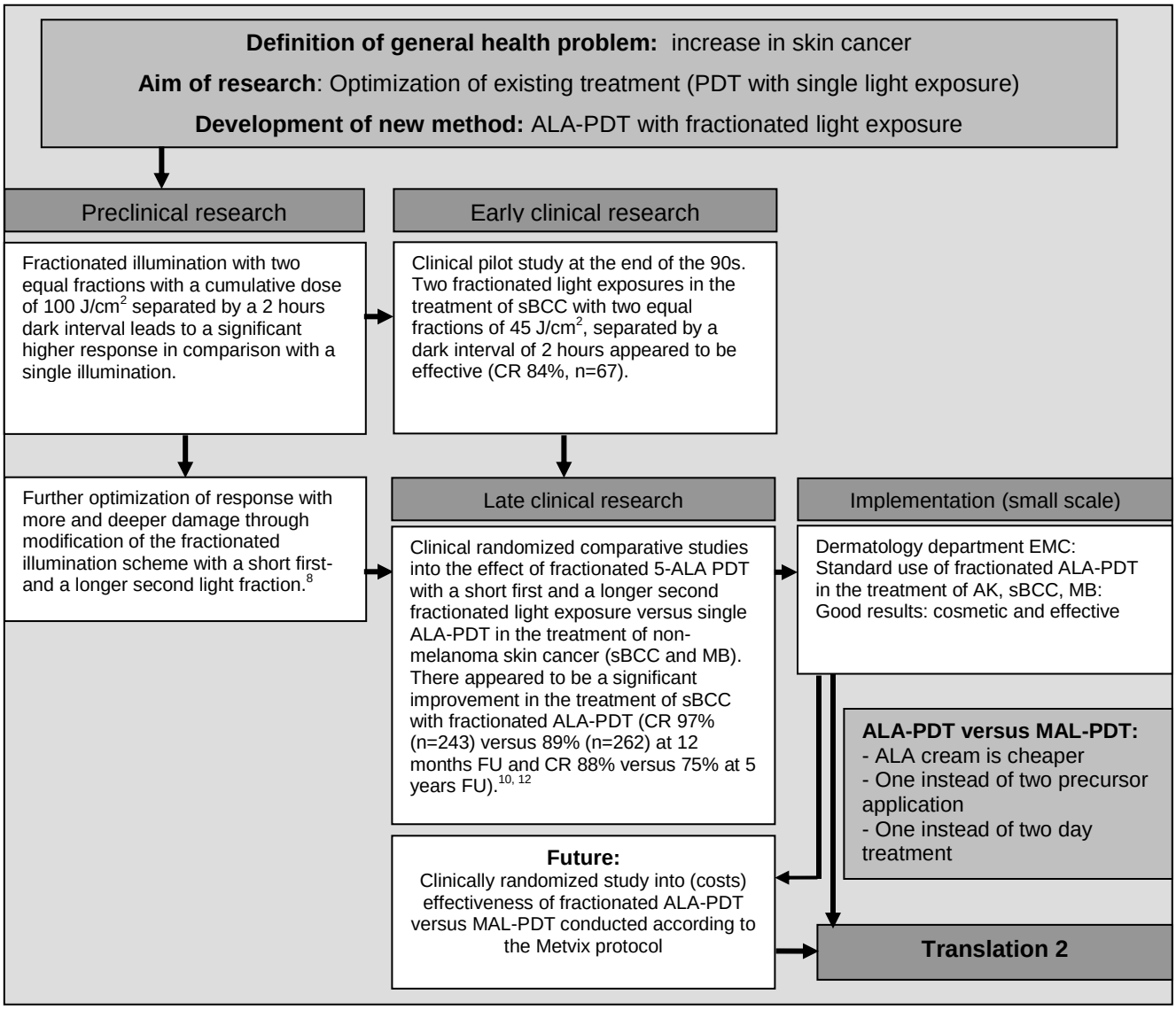

Figure 2: Translation step 1
(AK: Actinic keratosis, MB: Morbus Bowen, sBCC: Superficial basal cell carcinoma, CR: Complete response, FU: Follow-up).

The transfer of preclinical findings finally led to two randomized clinical trials (RCTs) in which fractionated ALA-PDT was compared with ALA-PDT using single illumination in the treatment of superficial BCC and Morbus Bowen, respectively. The complete response (CR) after a minimum follow up of 12 months appeared to be higher after fractionated ALA-PDT than after ALA-PDT using single illumination in both studies (97% versus 89% [P=0.002, log-rank test] and 88% versus 80% [P =0 .41 log-rank test]).[10,11] After 5 years follow-up the CR after fractionated ALA-PDT remained significant higher compared with single illumination (88% versus 75% [P=0.0002, log-rank test]).[12] Use of fractionation in MAL-PDT appeared not to improve effectiveness in a mouse model.[13] The CR for fractionated ALA-PDT is higher than that reported for repeated

single ALA- or MAL-PDT.[14] Differences in the effectiveness between MAL-PDT and fractionated ALA-PDT have not been investigated in RCTs. It was decided in 2005 to use fractionated ALA-PDT in daily practice at our department on the basis of these results.[10,11] Besides the high effectiveness of fractionated ALA-PDT, whereby a lower number of recurrences required treatment, extra costs and time were saved as compared to those in more often used MAL-PDT(according to the Metvix®-protocol): 1) in fractionated ALA-PDT, the treatment is on one day instead of two, 2) precursor formulation is applied once instead of twice and 3) the commercially available ALA-formulation is twice as cheap as Metvix® (€ 67/gram versus € 132/gram). An incidence of around 44,000 newly diagnosed BCC patients is expected in 2015 increasing to around 57,000 in 2020.[15] Subsequent BCCs in the same patient are not included in this, whereas, the chance of this is 29% is within 5 years.[15] An increase of CR of several percent and the lower costs of fractionated PDT shall result in a considerable reduction of treatment time and costs.

Translation 2

The next step, which must now be made, is T2: the actual implementation of fractionated ALA-PDT in the general health care, whereby care gain is to be achieved for the whole population. Implementation is defined as follows: the orderly and schematic introduction of renewals and changes of proven values with the aim that these occupy a structural place in the professional interventions.[16] Other research sciences are such as epidemiology, psychology, sociology, anthropology and economy are also involved for T2.[2] Hallmarks that predict a successful implementation are: advantage, compatible with the existing views, simplicity, tryout and apparentness.[17] It is important to rally support from the (intended) treatment providers, policy makers, industry and patients to realize implementation. What does one think of the already existing treatment (twice treatment with MAL-PDT). What does one come across and what would one like to see changed.

A good diagnostic analysis provides starting points for the approach to implementation. Hindering and enhancing factors for implementation should be identified and listed considering the various factors: the individual treatment provider, the social setting (patients, care providers) and structural factors (organization and financial conditions). An implementation strategy should be developed on the basis of the outcomes and directed at the treatment providers (refresher courses, feedback), patients (information, input by patients), care organization of material conditions (money, aids). A combination is probably more effective.[16] First steps have been taken in recent years to simplify the use of fractionated ALA-PDT for the intended treatment

providers. Since 2009, a pharmaceutical company manufactures an ALA-formulation for the Dutch market. In addition, scientific publications and lectures also attempt to rally support from the treatment providers. Nevertheless, the implementation of fractionated ALA-PDT appears not to succeed as yet, in spite of the proof. Various factors may play a role. The switch to fractionated-PDT probably has an insufficient advantage for the treatment provider. The treatment provider is generally the one who has to provide the maximum effort for the introduction of a new treatment in his/her department, whereas the advantages are namely for the patient (time saving and high effectiveness) and health care insurers (low costs). These two parties are probably inadequately informed. Moreover, the good marketing by the Metvix® supplier and the (financial) support of refresher courses and research possibly plays a role in the choice of the care provider. Aware or unaware influencing may occur. The lack of randomized comparative studies into (cost) effectiveness between MAL-PDT and fractionated ALA-PDT shall also not promote the implementation of fractionated ALA-PDT. A higher level of proof is a stronger incentive for both the treatment provider and the health care insurer to introduce a new treatment.

CONCLUSION

It appears from this example that translational research is a complex process in which a good T1 phase is imperative, but T2 is vital. The broad folding out of “evidence” obtained from scientific research into the daily practice is hindered by the complicated structure of our health care system. The ultimate success of translational research appears to depend on the willingness of the treatment providers to adapt their method to the preconditions which the health care providers, health care institutes and health care insurers want and can offer. Fortunately, more attention has been paid to T2 in the past years. ZonMW stimulates and subsidizes studies in which attention is paid to both T1 as well as T2 with the aim that valuable and costly findings from T1 may finally find their way into the daily practice. Extra stimulation for the T2 phase shall be required to quickly cash in on the effectiveness and the advantages of new treatments.

REFERENCES

1. Woolf SH. The meaning of translational research and why it matters. JAMA. 2008;299:211-213.
2. Olgilvie D, Craig P, Griffin S, et al. A translational framework for public health research. BMC public health, 2009;9:116-126.

3. Vries E de, Nijsten T, Louwman MW, Coebergh JW. Skin cancer epidemic in the Netherlands. Ned Tijdschr Geneeskd. 2009;153:A768.

4. Kloek J, Akkermans W, Beijersbergen, Henegouwen GM van. Derivatives of 5-aminolevulinic acid for photodynamic therapy: enzymatic conversion into protoporphyrin. Photochem Photobiol 1998;67:150-154.

5. De Bruijn HS, Meijers C, van der Ploeg-van den Heuvel A, et al. Microscopic localisation of protoporphyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl 5-aminolevulinate. J Photochem Photobiol B 2008;92:91-97.

6. Thissen MR, Kuijpers DI, Neumann HA. The wider application of photodynamic therapy in dermatology, Ned Tijdschr Geneeskd. 2005;149:232-237.

7. Veen N van der, Leengoed HL van, Star WM. In vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. Br J Cancer 1994;70:867-872.

8. Robinson DJ, Bruijn HS de, Wolf WJ de, Sterenborg HJCM, Star WM. Topical ALA-photodynamic therapy of hairless mouse skin using two fold illumination schemes: PpIX fluorescence, photobleaching and biological effects. Photochem Photobiol 2000;72:794-802.

9. Robinson DJ, Bruijn HS de, Star WM, Sterenborg HJCM. Dose and timing of the first light fraction in two-fold illumination schemes for topical ALA-mediated photodynamic therapy of hairless mouse skin. Photochem Photobiol, 2003;77:319-23.

10. Haas ERM de, Kruijt B, Sterenborg HJCM, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. J Invest Dermatol 2006;126:2679-2686.

11. Haas ERM de, Sterenborg JJCM, Neumann HAM, Robinson DJ. Response of Bowen's disease to ALA-PDT using a single and 2-fold illumination scheme. Arch Dermatol. 2007;143:264-265.

12. Vijlder HC de, Sterenborg HJCM, Neumann HAM et al. Light fractionated significantly improves the response of superficial basal cell carcinoma to ALA-PDT: five-year follow-up of a randomized prospective trial. Acta Derm Venereol. 2012;92:641-647.

13. Bruijn HS de, Haas ERM de, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated photodynamic therapy in normal mouse skin, Photochem Photobiol Sc. 2007;6:1325-1331.

14. Braathen, LR, Szeimies RM, Basset-Seguin N, et al. Guidelines on the use of photodynamic therapy for nonmelanoma skin cancer: An international consensus. J. Am Acad Dermatol, 2007;1:125-143.

15. Flohil SC, Koljenović S, de Haas ER et al. Cumulative risks and rates of subsequent basal cell carcinomas in the Netherlands Br J Dermatol. 201;165:874-881.

16. Bron: Zorg Onderzoek Nederland. Met het oog op toepassing, beleidsnota, implementatie ZON 1997-1999, Den Haag 1997.

17. ZonMW-site: zonmw.nl/nl/implementatie/kennis-delen/literatuur.

18. Wensing M, Splunteren P van, Hulscher M, Grol R. Praktisch Nieuw. Implementatie van vernieuwing in de gezondheidszorg, van Gorcum & Comp, Assen, 2000.

9 General Discussion

H. C. de Vijlder

The main aims of the investigations described in this thesis were three-fold. Firstly, the long-term efficacy of fractionated ALA-PDT in the treatment of superficial BCCs. Secondly, the mechanism responsible for the increased efficacy of fractionated PDT and thirdly the role of MAL-PDT in the treatment of Bowen's disease. In addition, the genetic aberrations within the pigmentation pathway, causing albinism, and the association with UV-induced skin cancer are dealt with in details.

Clinical response to and mechanism of light fractionated ALA-PDT

Photodynamic therapy (PDT) using the porphyrin precursors ALA and MAL is widely accepted as a treatment option for superficial BCCs, Bowen's disease and actinic keratosis.[1] It has also been used to treat a variety of non-malignant skin diseases and extra-cutaneous malignancies in the oral cavity, the genitourinary tract and the gastrointestinal tract. Whereas the treatment of superficial non-melanoma skin cancers (NMSCs) with PDT has good short-term results, long-term clinical results are sparse, are less impressive and show a considerable variation in the recurrence rates. A strategy to increase the effectiveness of PDT is by splitting the illumination scheme into two light fractions. Interest in light fractionated ALA-PDT arose almost 2 decades ago by the clinical observation that PpIX re-appeared in the time after the therapeutic illumination [W.M. Star, personal communication]. The initial rationale behind light fractionation using a long dark interval was to utilise this additional PpIX for PDT. The results obtained in the first pre-clinical studies using this type of light fractionation were promising.[2,3] Subsequent studies aimed at increasing the efficacy and elucidating the mechanism underlying the increased response led to some important conclusions without providing a full understanding of how they related to the increased response *in-vivo*. It was shown that the increased response to light fractionation was the highest when a low dose fraction was followed by a high dose fraction, separated by a dark interval of two hours. The choice of the fluence for the first fraction was critical and a high fluence for the second light fraction and a dark interval of more than 90 minutes were required for maximum tissue response.[3,4] These pre-clinical results were confirmed in a randomized clinical trial comparing ALA-PDT using a single fluence (75 Jcm^{-2}) with a two-fold illumination (20 + 80 Jcm^{-2}) in the treatment of superficial BCCs. An interim analysis at 12 months demonstrated a statistically significant improvement in complete response rate for light fractionated PDT.[5] The results of the long- term follow-up presented in **chapter 2** showed that this statistically significant improvement in the complete response rate was maintained after 60 months of follow-up. This study is one of the few large scale long-term randomized clinical studies investigating PDT response and the first to show that efforts to optimize PDT may lead to a significant increase in the long-term clinical response. The

increase is not simply a statistical difference, but is a significant result for the patients. Light fractionated ALA-PDT requires approximately 1 in 10 patients to be re-treated after 5 years as compared with 1 in 4 patients using the traditional ALA-PDT.

Pre-clinical studies in which the mechanism of the increased response to fractionated ALA-PDT was investigated are described in **chapters 3 and 4**. This was done by studying the differences between ALA and MAL since it was shown that the increase in response to light fractionated PDT did not occur when MAL-PDT was used.[6] Previous studies investigating the difference between ALA and MAL led to the conclusion that the vasculature in the dermis may be involved in the difference in response of normal mouse skin following ALA- and MAL-PDT.[7,8] Therefore, we investigated differences in tissue distribution of PpIX and determined the level of co-localisation of PpIX with the endothelial marker CD31 using confocal microscopy in frozen sections of tissues obtained from mice applied with topical MAL or ALA **(chapter 3**). No significant difference in the absolute intensity of PpIX fluorescence following MAL or ALA application was observed in the epidermis. However, in the dermis that was used for analysis, the distribution of PpIX showed a significantly higher level of co-localisation in CD31 stained vasculature 4 hours after the application of ALA as compared with that after the application of MAL ($p<0.01$). This observation is the first direct indication that the distribution of PpIX in the vasculature is different for various porphyrin precursors. This analysis did not attempt to take into account any potential influence of the depth of vessels on the level of co-localisation of PpIX and the endothelial cells in the dermis. Given the penetration profiles of ALA and MAL and the probable removal of ALA via the local vasculature in the dermis, it is possible that vessels close to the surface, nearer the epidermis, will have a higher level of co-localisation between PpIX and CD31 for both ALA and MAL. Given the difficulty of accurately determining the depth of individual groups of vessels in non-vertical sections of frozen tissue, we developed an *in-vivo* model using intra-vital confocal microscopy in which we were able to study the differences in spatial distribution of PpIX at different depths in the skin of mice after ALA or MAL application (**chapter 4).** Moreover, it was also possible to study the acute vascular effects of photodynamic therapy (PDT) in this model. ALA or MAL were topically applied for four hours. This resulted in high PpIX fluorescence intensities in the epidermis and lower, but substantial PpIX fluorescence intensities in the dermis and sub-cutis for both PpIX precursors. The subcutaneous PpIX fluorescence was higher for ALA as compared with that for MAL. This observation was remarkable since it is generally assumed that PpIX synthesis induced after ALA or MAL application does not extend much further than the epidermis because of poor skin penetration.[9] The differences in dermal and subcutaneous PpIX fluorescence intensity between MAL and ALA may be explained by the more lipophilic character of MAL reducing

the penetration through the epidermis. MAL penetrates into cells more quickly and may be suspended at high concentration within the lipid membrane bilayer of cells in the epidermis and higher dermis as compared with that with ALA. The more hydrophilic character of ALA may allow better penetration through the epidermis into the dermis and the sub-cutis. The distribution of PpIX in the sub-cutis was not homogeneous, with a preference for the (peri)vascular regions, especially after ALA application. Subsequently the effect of the PpIX distribution on the PDT-induced vascular response was investigated. Both low (10 Jcm^{-2}) and high (100 Jcm^{-2}) fluence PDT induced acute vascular constriction in the dermis and the sub-cutis. The vasoconstriction after ALA- PDT was more pronounced as compared with that after MAL-PDT. Moreover, vascular leakage in the dermal vessels was observed only after ALA-PDT. This leakage indicates endothelial damage. PDT induces an increase in endothelial permeability, by activating RhoA, causing the breakdown of the cortical actin band to form stress fibres and then rearranging VE-cadherin, all of which lead to changes in the endothelial cell morphology.[10] The thinner vessel walls in the dermis, designed for optimal exchange by diffusion of O_2 and CO_2 or transcellular transport for other molecules, are probably better permeable for ALA, resulting in higher PpIX concentration, making the capillary walls more vulnerable than the thicker arteriole walls. This may possibly explain why we did not observe leakage in the subcutaneous arterioles. The endothelial damage in the dermis and the vascular effects in the sub-cutis may explain the formation of more acute oedema in the first few hours after fractionated ALA-PDT as observed in a previous study by our group.[6] This study supports our findings that vascular responses play a role in topical PDT. The fact that vascular damage in the dermis and more vasoconstriction in the sub-cutis are already induced by low fluences, corresponding with the first light fraction of the fractionated illumination scheme supports our hypothesis that the endothelial cells are involved in the increased response of fractionated ALA-PDT.

We suggest that the low PpIX concentration observed in dermal and subcutaneous vasculature after the topical application of ALA is in the optimal range to induce vascular effects after a first low fluence light fraction. The low PpIX concentrations in the endothelial cells are important since de Bruijn et al.[11] recently reported in an *in-vitro* study that the concentration of PpIX at the time of the first light fraction determined the efficacy of light fractionated PDT. Enhanced cell death in response to light fractionated ALA-PDT was only obtained in cells incubated with low concentrations of ALA. A low ALA application results in an accumulation of PpIX mainly in the mitochondria. De Bruijn et al.[11] suggested that the delivery of a small PDT dose followed by a dark interval will bring the tumour cells in a pre-lethal phase making them more vulnerable to apoptosis or autophagy. This effect was not observed after incubation with high ALA concentration, possibly overwhelming the cells

leading to necrosis instead of apoptosis. The second light fraction activates PpIX, that in turn generates ROS which attack mitochondrial lipids and factors weakening the mitochondrial membrane.[12] This triggers cytochrome c release into the cytosol and activation of the caspase cascade and apoptotic cell death.[13,14,15] The low dermal and subcutaneous PpIX concentration observed after topical MAL application may be too low to induce sufficient vascular effects. The profit of bringing the cells in pre-lethal state by the first illumination of fractionated ALA –PDT may be compared with pre-PDT treatments that destabilizes the cells such as pre-conditioning with differentiation-modulating agents as methotrexate or calcitriol, which promote PDT induced apoptotic cell death.[16,17] However, modulating the delivery of light is a relatively simple practical approach that is easily achievable in a clinical setting.

The results described **in chapters 2-4** are obviously important for superficial non-melanoma skin malignancies and pre-malignancies that are currently indicated for PDT. It may also improve efficacy of thicker non-melanoma skin cancer lesions and efficacy of extra-cutaneous malignancies. The efficacy of ALA- and MAL-PDT in the treatment of nodular BCCs was studied, but the recurrence rates were high.[18,19] Since it was observed that fractionated ALA-PDT leads to a deeper histological damage in preclinical studies, combined with the result of our long-term follow-up response rates of fractionated PDT, higher response rates in the treatment of nodular BCCs may be expected. Surgical excision is considered the gold standard in the treatment of nodular BCCs. However, healing time, cosmetic outcome and cost- effectiveness are more favourable for PDT. A drawback of PDT is the stinging and burning pain that may accompany treatment. The severity of pain varies from a transient discomfort to severe pain. However, this may be reduced by applying low fluence rate PDT. Cottrell et al.[20] performed a systematic clinical investigation on the effect of fluence rate on ALA-PDT efficiency and pain and they observed that lowering the fluence rate reduced the pain during illumination, while maintaining the efficacy in the treatment of superficial BCCs. An *in-vivo* study showed that the increased efficacy of light fractionated ALA-PDT was maintained when using low fluence rate PDT.[21]

MAL-PDT versus excision surgery

The use of MAL in superficial non-melanoma skin cancer is described in **chapters 5 and 6**. MAL-PDT following the Metvix® protocol was compared with surgical excision in the treatment of Bowen's disease (BD). This study is the only (published) prospective randomized clinical study comparing topical PDT using porphyrin precursors and simple excision surgery, considered the gold standard treatment for BD. The results of our study showed a significantly higher efficacy for surgical excision as compared with that for MAL-PDT with a complete response rate of 96.0% for surgical excision and 69.6% for MAL-PDT

after 18 months of follow-up (P = 0.018, Fisher exact test). The main advantages of surgery as compared with MAL-PDT in our study was obviously the significantly higher efficacy and the securing of histologically free excision margins, so that irradically excised lesions could be re-excised directly after histological examination. However the cosmetic and functional outcome at some body sites, the risk of post-surgical bleeding, poor vascularity and prolonged or complicated wound healing may be a limitation for surgery, especially for large BD lesions. BD lesions in several trials were treated more than once to achieve higher complete responses.[22,23,24,25] In our trial MAL-PDT was repeated at one week, according to the Galderma Metvix® protocol. A higher efficacy in the treatment of BD was not achieved despite this approach. A number of factors may limit the response of BD to MAL-PDT. The effectiveness of PDT in large lesions may be limited by the inability of the vasculature in and around the illumination area to supply oxygen for the photodynamic process. The utilisation of oxygen during PDT may be increased by reducing the fluence rate thereby lowering the demand for oxygen. It is well documented that using a lower fluence rate, while maintaining the fluence, results in more efficient usage of the available oxygen in tissue.[26,27,28,29,30,31] Lowering the fluence rate may be an interesting modification of the existing MAL-PDT illumination scheme for the treatment of BD. The use of fractionated ALA-PDT increases the complete response in the treatment of BD as compared with ALA-PDT using a single illumination.[25] A complete response rate of 80% was achieved in the single illumination group at 12 months as compared with 88% in the fractionated group. Although higher, this increase did not reach statistical significance. Given the potential limitations of the supply of oxygen in large lesions, the combination of fractionated ALA-PDT and low fluence rate PDT may be an alternative approach.[21] Moreover, the penetration of ALA may be improved in order to reach optimum ALA and PpIX concentrations in the dermal and subcutaneous endothelial cells to increase the efficacy of fractionated PDT further. A liposomal formulation of ALA was recently introduced. Liposomal formulations are known to penetrate deeper into the epidermis,[32] whether it results in higher concentrations of ALA and thus PpIX in the dermal and subcutaneous endothelial cells needs further investigation. To date we have not been able to increase the efficacy of MAL-PDT using light fractionation. However, it would be interesting to investigate whether MAL in a liposomal formulation would result in higher dermal and subcutaneous concentration. If dermal and subcutaneous PpIX fluorescence intensities are higher and are co-localised within the vasculature, then an increased efficacy may also be achieved by using fractionated PDT for MAL.

Populations at risk for developing skin cancer risk

Skin colour is one of the risk factors for skin cancer. Specifically, fair skin, red and blond hair, green and blue eyes in various studies have been linked to increased risk of skin cancers. Many of the polymorphisms in genes within the pigmentation pathway show association with the risk on skin cancers.[33,34,35,36,37] Oculocutaneous Albinism (OCA) comprises a group of genetic disorders with various mutations in the pigmentation pathway genes. Mutations in genes encoding for tyrosinase (OCA1A and OCA1B), P-protein (OCA2), tyrosinase-related-protein 1 (OCA3) and MATP (OCA4) have been described.[38] OCA patients are highly susceptible to UV-induced skin cancers presumably especially those producing low amounts of pheomelanine. So differences in the sensitivity to skin carcinogenesis may be expected, depending on the mutations in OCA **(chapter 7).** Therefore the various mutations in the OCA subtypes and phenotypic consequences may provide a model to study the role of these pigmentation genes and encoding proteins in the development of skin cancers.

Implementation of fractionated ALA-PDT

The rising incidence of skin cancer, particularly NMSC, impose an ever increasing care burden on an increasing number of physicians indicating that optimized treatment protocols such as fractionated PDT are relevant**.** An increase in the complete response of several percent will reduce the number of recurrences considerably and thus the patient burden and the extra costs for re-treatment. In a recent comparative cost minimization study MAL (Metvix®) PDT, imiquimod (Aldara®) and 5-fluorouracil (Efudix®) were compared in the treatment of superficial BCCs. The cost minimization analysis showed that 5-fluorouracil was a cheaper treatment for superficial BCCs as compared with imiquimod and MAL-PDT.[39] It is highly likely that in the near future health care insurers will increasingly reimburse only the most cost-effective treatments. Although there are no studies available on the cost-effectiveness of fractionated ALA-PDT to date, we believe that fractionated ALA-PDT is more cost-effective than at least MAL-PDT, based on the lower costs per patient and the high efficacy for fractionated ALA-PDT in our long- term follow-up study (**chapter 8**). It would be interesting to investigate whether fractionated ALA-PDT is more effective and cost-effective than 5-fluorouracil. An advantage of fractionated PDT over 5-fluorouracil is the relatively short duration of treatment and the adherence rate due to the clinical setting.

REFERENCES

1. Lee Y, Baron ED. Photodynamic Therapy: Current Evidence and Applications in Dermatology *Seminars in cutaneous medicine and surgery* 2011;**30**:199-209.

2. van der Veen N, van Leengoed HLLM, Star WM. In vivo fluorescence kinetics and photodynamic therapy using 5-aminolaevulinic acid-induced porphyrin: increased damage after multiple irradiations. *Br J Cancer* 1994;**70**:867-872.

3. van der Veen N, Hebeda KM, de Bruijn HS, Star WM. Photodynamic effectiveness and vasoconstriction in hairless mouse skin after topical 5-aminolevulinic acid and single- or two-fold illumination. *Photochem Photobiol* 1999;**70**:921-929.

4. de Bruijn HS, van der Veen N, Robinson DJ, Star WM. Improvement of systemic 5-aminolevulinic acid-based photodynamic therapy in vivo using light fractionation with a 75-minute interval. *Cancer Research* 1999;**59**:901-904.

5. de Haas ERM, Kruijt B, Sterenborg HJCM, et al. Fractionated illumination significantly improves the response of superficial basal cell carcinoma to aminolevulinic acid photodynamic therapy. *J Invest Dermatol* 2006;**126**:2679-2686.

6. de Bruijn HS, de Haas ER, Hebeda KM, et al. Light fractionation does not enhance the efficacy of methyl 5-aminolevulinate mediated PDT in normal mouse skin. *Photochem Photobiol Sci* 2007;**6**:1325-1331.

7. de Bruijn HS, Meijers C, van der Ploeg-van den Heuvel A, et al. Microscopic localisation of protoporphyrin IX in normal mouse skin after topical application of 5-aminolevulinic acid or methyl 5-aminolevulinate. *J Photochem Photobiol B* 2008;**92**:91-97.

8. Moan J, Ma LW, Juzeniene AV, et al. Pharmacology of protoporphyrin IX in nude mice after application of ALA and ALA esters. *Int J Cancer* 2003;**103**:132-135.

9. Schulten R, Novak B, Schmitz B, Lubbert H. Comparison of the uptake of 5-aminolevulinic acid and its methyl ester in keratinocytes and skin. *Naunyn-Schmiedeberg's Arch Pharmacol* 2012:**385**:969-979.

10. Ota H, Matsumura M, Miki N, Minamitami H. Photochemically induced increase in endothelial permeability regulated by RhoA activation. *Photochem Photobiol Sci* 2009;**8**:1401-1407.

11. de Bruijn HS, Casas A, Di Venosa G, et al. Light fractionated ALA-PDT enhances therapeutic efficacy in-vitro; the influence of PpIX concentration and illumination parameters. *Photochem Photobiol* 2012; Oct 29 (Epub ahead of print).

12. Kriska T, Korytowski W, Girotti AW. Role of mitochondrial cardiolipin peroxidation in apoptopic photo-killing of 5-aminolevulinate-treated tumor cells. *Arch Biochem Biophys* 2005;**433**:435 446.

13. Kessel D, Vicente MG, Reiners JJ. Initiation of apoptosis and autophagy by photodynamic therapy. *Lasers Surg Med* 2006;**38**:482-488.

14. Buyaert E, Dewaele M, Agostinis P. Molecular effectors of multiple cell death pathways initiated by photodynamic therapy *Biochim Biophys Acta* 2007;**1776**:86-107.

15. Ji Z, Yang G, Vasovic V, et al. Subcellular localization pattern of protoporphyrin IX is an important determinant for its photodynamic efficiency of human carcinoma and normal cell lines. *J Photochem Photobiol B: Biology* 2006;**84**:213-220.

16. Anand S, Honari G, Hasan T et al. Low-dose methotrexate enhances aminolevulinate-based photodynamic therapy in skin carcinoma cells. *In vitro* and *in vivo. Clin Cancer Res* 2009;**15**:3333 – 3343.

17. Anand S, Wilson C, Hasan T, Maytin EV. Vitamin D3 enhances the apoptopic response of epithelial tumors to aminolevulinate-based photodynamic therapy. *Cancer Res* 2011;**71**:6040–6050.

18. Rhodes LE, de Rie MA, Leifsdottir R, et al. Five-year follow-up of a randomized, prospective trial of topical mehthyl aminolevulinate photodynamic therapy vs. surgery for nodular basal cell carcinoma. *Arch Dermatol* 2007;**143**:1131-1136.

19. Foley P, Freeman M, Menter A, et al. Photodynamic therapy with methyl aminolevulinate for primary nodular basal cell carcinoma: results of two randomized studies. *Int J Dermatol* 2009;**48**:1236-1245.

20. Cottrell WJ,Paquette AD, Keymel KR, et al. Irradiance-dependent photobleaching and pain in d-Aminolevulinic acid-photodynamic therapy of superficial basal cell carcinomas. *Clin Cancer Res* 2008;**14**:4475-4483.

21. Middelburg TA, Van Zaane F, De Bruijn HS, et al. Fractionated illumination at low fluence rate photodynamic therapy in mice. *Photochem Photobiol* 2010;86:1140-1146.

22. Morton CA, Whitehurst C, Moore JV, MacKie RM. Comparison of red and green light in the treatment of Bowen's disease by photodynamic therapy. *Br J Dermatol* 2000;**143**:767-772.

23. Morton CA, Whitehurst C, Moseley H, et al. Comparison of photodynamic therapy with cryotherapy in the treatment of Bowen's disease. *Br J Dermatol* 1996;**135**:766-771.

24. Salim A, Lehman JA, McColl JH, et al. Randomized comparison of photodynamic therapy with topical 5-fluorouracil in Bowen's disease. *Br J Dermatol* 2003;**148**:539-543.

25. de Haas ERM, Sterenborg JJCM, Neumann HAM, et al. Response of Bowen's disease to ALA-PDT using a single and 2-fold illumination scheme. *Arch Dermatol* 2007;**143**:264-265.

26. Langmack K, Mehta R, Twyman P, Norris P. Topical photodynamic therapy at low fluence rates theory and practice. *J Photochem Photobiol B* 2001;**60**:37-43.

27. Finlay JC, Conover DL, Hull EL, Foster TH. Porphyrin bleaching and PDT-induced spectral changes are irradiance dependent in ALA-sensitized normal rat skin in vivo. *Photochem Photobiol* 2001;**73**:54-63.

28. Robinson DJ, de Bruijn HS, van der Veen N, et al. Fluorescence photobleaching of ALA-induced protoporphyrin IX during photodynamic therapy of normal hairless mouse skin: the effect of light dose and irradiance and the resulting biological effect. *Photochem Photobiol* 1998;**67**:140-149.

29. Foster TH, Murant RS, Bryant RG, et al. Oxygen consumption and diffusion effects in photodynamic therapy. *Radiat Res* 1991;**126**:296-303.

30. Sitnik TM, Henderson BW. The effect of fluence rate on tumor and normal tissue responses to photodynamic therapy. *Photochem Photobiol* 1998;**67**:462-466.

31. Henderson BW, Busch TM, Vaughan LA, et al. Photofrin photodynamic therapy can significantly deplete or preserve oxygenation in human basal cell carcinomas during treatment, depending on fluence rate. *Cancer Res* 2000;**60**:525-529.

32. de Leeuw J, de Vijlder HC, Bjerring P, Neumann HAM. Liposomes in dermatology today. *J Eur Acad Derm Venereol* 2009;**23**:505-516.

33. Sturm RA, Duffy DL, Zhao ZZ, et al. A single SNP in an evolutionary conserved region within intron 86 of the HERC2 gene determines human blue-brown eye color. *Am J Hum Genet* 2008;**82**:424–431.

34. Veierod MB, Adami HO, Lund E, et al. Sun and solarium exposure and melanoma risk: effects of age, pigmentary characteristics, and nevi. *Cancer Epidemiol. Biomarkers Prev* 2010;**19**:111-120.

35. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. *Int J Cancer* 2008;**122**:2753–2760.

36. Gerstenblith MR, Shi J, Landi MT. Genome-wide association studies of pigmentation and skin cancer: a review and meta-analysis. *Pigment Cell Melanoma Res* 2010;**23**:587-606.

37. Duffy DL, Zhao ZZ, Sturm RA, et al. Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. *J Invest Dermatol* 2010;**130**:520–528.

38. Scherer D, Kumar R. Genetics of pigmentation in skin cancer - A review. *Mutation Research* 2010;**705**:141-153.

39. Arits A, Essers B. Mosterd K et al. Photodynamic therapy versus topical imiquimod versus topical 5 fluorouracil for treatment of superficial basal cell carcinoma. a single blind, noninferiority, randomized controlled trial and cost-effectiveness analysis, Thesis, A. Arits, Maastricht University, 2013

10 Summary
Samenvatting

SUMMARY

The general introduction of this thesis is described in **Chapter 1**. Non-melanoma skin cancer (NMSC) is the most common cancer in Caucasians and its incidence continues to rise faster than that of any other cancers. In view of this increasing incidence, (cost)-effective treatment modalities with optimum functional and cosmetic results are imperative. Topical photodynamic therapy (PDT) is a non-invasive and effective treatment modality for superficial non-melanoma skin (pre-)malignancies with good cosmetic and functional results. The mechanism of PDT using the porphyrin precursors, aminolaevulinic acid (ALA) and its ester derivative methyl-aminolaevulinic acid (MAL) are described. An overview of several ways to optimize the efficacy of topical PDT including that of ALA-PDT by light fractionation is also presented.

The studies described in **Chapter 2,** focussed on the clinical long- term response of fractionated ALA-PDT with a two-fold illumination scheme as compared with a traditional single illumination scheme in the treatment of superficial basal cell carcinoma (BCC). An interim analysis of the complete response rate time-to-event analysis at 12-months showed a significantly better outcome for fractionated ALA-PDT. The 5-year follow-up data analysis of our randomized clinical trial is also dealt with here. This study represents one of the few reported large scale long-term randomized clinical studies on topical PDT. The complete response rate in the group treated with two-fold illumination was 88% after 5 years follow-up as compared with the complete response rate of 75% in the group treated with single illumination ($p = 0.0002$, log-rank test). This increase in the clinical response was not simply a statistical difference, but it was a significant result for the patients. Light fractionated ALA-PDT required approximately 1 in 10 patients to be retreated after 5 years as compared with 1 in 4 using the traditional ALA-PDT.

The mechanism behind the increased effectiveness of two-fold fractionated ALA-PDT was the focus of investigations described in **Chapters 3 and 4**. In contrast to ALA-PDT, it was shown in an earlier pre-clinical study that using the two-fold illumination scheme with MAL did not lead to an increased response as compared with single illumination. An important difference between ALA and MAL is their tissue distribution because of their different biophysical and biochemical characteristics. Based on earlier studies it was suggested that this difference in tissue distribution, especially the difference in endothelial cell involvement, may play a role in the difference in response to fractionated PDT. Therefore, we examined the differences in the distribution of PpIX in blood vessels after the application of ALA or MAL in histological slides (chapter 3) and in an *in-vivo* window chamber model (chapter 4). We noted that there was a significantly higher degree of co-localization of PpIX in the vasculature after ALA application as compared with MAL ($p<0.01$). This was the first direct indication that the distribution of PpIX in the vasculature was different for different porphyrin precursors. The

influence of the PpIX concentration in the endothelial cells on the vascular response during and after PDT was also investigated in the *in-vivo* window chamber model. Acute vascular constriction in the dermis and subcutis was obtained by low and high fluence PDT after ALA and MAL application. The effect was more pronounced for ALA. Vascular leakage was only observed in the dermal vessels after ALA-PDT, indicating endothelial damage. The fact that vascular damage in the dermis and more vasoconstriction in the subcutis were already induced by low fluences for ALA-PDT, corresponding with the first light fraction of the fractionated illumination scheme, supported our hypothesis that the endothelial cells were involved in the increased response of fractionated ALA-PDT.

The use of MAL-PDT in the treatment of non-melanoma skin cancer and acne vulgaris is described in studies in **Chapters 5 and 6.** In chapter 6, the clinical response and the cosmetic outcome of MAL-PDT was compared with surgical excision (gold standard therapy) in the treatment of Bowen's disease (BD) in a randomized clinical trial. After 18 months follow-up, the relative complete response rate was significantly higher for excision (96.0%) as compared with MAL-PDT (69.6%) (P=0.018, Fisher exact test). Cosmetic outcome was better for MAL-PDT. Analyses of the results in the MAL-PDT group showed that lesion size may be a predictive factor for recurrences. We noted an association of relapse with larger diameter. Lesions equal to or larger than 20 mm showed border line significantly lower complete response as compared with lesions that were smaller than 20 mm in diameter (43.9% versus 81.2%, P=0.06, Fisher exact test). The advantage of non-invasive therapies such as PDT is particularly important for larger lesions at poorly vascularized locations that are associated with prolonged wound healing. Therefore, it is disappointing that the majority of recurrences occur in these large lesions. The complete response rates of MAL-PDT for BD were comparable with reported results in the literature, but further improvements remain essential.

The differences in the risk for developing skin cancer within the different types of oculocutaneous albinism were investigated in a literature study, described in **Chapter 7**. Although it is known that albinos have a high risk to develop skin cancer, the current literature fails to provide convincing evidence for the differences in the risks between the various types of albinism. Since pheomelanin induces ROS, which renders especially red-haired people more susceptible to skin cancer, we concluded that albinos having pheomelanin in the keratinocytes must be more susceptible to skin cancer than albinos without any melanin. An indication that this may be the case is that the white skin of those with vitiligo, devoid of melanocytes and thus unable to produce any melanin is resistant to melanomas, basal cell- and squamous cell carcinomas.

Optimization of treatment modalities for non-melanoma skin cancer, such as PDT, is highly important in reducing the demands on the public health care system. Translational research

offers the possibility to implement such a reduction. This type of research may be divided into T1, in which the knowledge of the disease mechanism is converted into new methods - the 'bench to bedside' component, and T2 component in which clinical studies are translated into the daily practice. Some of the pitfalls in the implementation (T2) of the optimized two-fold illumination scheme for ALA-PDT in the daily practice are described in **Chapter 8.**

The most important results of the above mentioned studies are discussed in the context of the current concepts in the literature and future perspectives in the general discussion presented in **Chapter 9.**

SAMENVATTING

In **Hoofdstuk 1** wordt de achtergrond gegeven voor de studies beschreven in dit proefschrift met als titel: “Nieuwe inzichten in fotodynamische therapie gebruikmakend van voorlopermoleculen van protoporfyrine IX”. Fotodynamische therapie (PDT) is een behandeling met zichtbaar licht voor (niet-melanoom) huidkanker. Huidkanker is de meest voorkomende kanker onder de blanke bevolking en wordt voornamelijk veroorzaakt door blootstelling aan zonlicht. De incidentie (het voorkomen) neemt snel toe en zal in de nabije toekomst leiden tot een grotere belasting van de gezondheidzorg. Het toepassen van effectievere en kostenbesparende behandelingen is dan ook noodzakelijk om deze belasting te verminderen.

Lokale PDT wordt sinds tientallen jaren toegepast in de behandeling van oppervlakkige (niet-melanoom) huidkanker. De korte termijn effecten zijn goed, maar langere termijn effecten vallen tegen. Bij lokale PDT wordt 5-aminolevulinezuur (ALA) of methyl-aminolevulinaat (MAL) op de huidtumor aangebracht. Deze beide voorlopermoleculen worden omgezet in het lichtgevoelige protoporfyrine IX (PpIX). In huidkanker is meer PpIX-vorming dan in de gezonde huid. Belichting van PpIX in de huid resulteert in de vorming van geactiveerd zuurstof. Dit geactiveerde zuurstof geeft schade en vernietigt de huidkanker.

Er is op verschillende manieren geprobeerd de doelmatigheid van PDT te verbeteren. Uit proefdieronderzoeken en klinische studies is bijvoorbeeld gebleken dat belichting in twee fracties in plaats van eenmalige belichting betere resultaten geeft bij ALA-PDT. Deze gefractioneerde belichting werkt het best als er eerste een korte lichtfractie wordt gegeven gevolgd door een langere lichtfractie na twee uur.

In een gerandomiseerde klinische studie beschreven in **hoofdstuk 2** is het lange termijn effect van gefractioneerde ALA-PDT (met korte en lange belichtingen van respectievelijk 20 en 80 J/cm^2) onderzocht in vergelijking met ALA-PDT met enkelvoudige belichting (75 J/cm^2). Analyse van de behandelresultaten na vijf jaar liet een statistisch significant beter resultaat zien in de groep behandeld met gefractioneerde belichting (88%) in vergelijking met de groep, die behandeld was met enkelvoudige belichting (75%) (p=0.0002, log-rank test). Dit betekent dat na 5 jaar slechts 1 op de 10 patiënten uit de gefractioneerde ALA-PDT groep opnieuw behandeld moest worden in vergelijking met 1 op de 4 patiënten uit de enkelvoudige belichtingsgroep.

De studies naar het mechanisme achter de toegenomen effectiviteit van gefractioneerde ALA-PDT worden beschreven in de **hoofdstukken 3 en 4.** Uit eerdere proefdieronderzoeken bleek dat, in tegenstelling tot ALA, toepassing van het gefractioneerde belichtingsschema bij MAL geen verbetering gaf ten opzichte van enkelvoudige belichting. Het vermoeden was dat een verschil in de hoeveelheid PpIX in de vaatwanden na het aanbrengen van ALA of MAL verantwoordelijk is voor het verschil in respons op gefractioneerde PDT. Om de rol van de

vaatwanden te onderzoeken hebben we de co-localisatie (het samenvallen) van PpIX met de vaatwanden 4 uur na het aanbrengen van ALA of MAL op muizenhuid onderzocht (**hoofdstuk 3**). Om dit te onderzoeken hebben we vier uur na het aanbrengen van ALA of MAL, muizenhuid gebiopteerd en vervolgens in dunne plakjes (coupes) gesneden. Door aankleuring van de vaatwanden met een vaatmarker konden we met een microscoop bepalen hoeveel PpIX samenviel met de vaatmarker. PpIX viel in significant hogere mate samen met de vaatwanden na het aanbrengen van ALA in vergelijking met MAL ($p<0.01$). Omdat het in de coupes niet mogelijk was het effect van PDT op de vaatwanden te bestuderen hebben we een proefdiermodel ontwikkeld waarin de vaten van de huid in een levende muis goed zichtbaar waren (zgn. window-chamber-model) (**hoofdstuk 4**). Vier uur na het aanbrengen van ALA was de hoeveelheid PpIX in en rondom de vaten hoger dan na het aanbrengen van MAL. Na het geven van een kleine hoeveelheid licht knepen de vaten voor beide groepen samen. Bij ALA was de mate van vasoconstrictie (het samenknijpen van vaten) en de correlatie tussen PpIX concentratie en vasoconstrictie hoger. Daarnaast trad er tijdens ALA-PDT vaatlekkage op, duidend op vaatschade. Vaatlekkage werd niet gezien bij MAL-PDT. Deze studie toont aan dat vaten betrokken zijn bij lokale PDT en dat dit effect veel groter is voor ALA-PDT dan voor MAL-PDT. Het feit dat het vaateffect al optreedt na een korte belichting, overeenkomend met de eerste lichtfractie (5-10J/cm^2) van het gefractioneerde schema, ondersteunt onze hypothese dat de vaatwanden betrokken zijn bij de toegenomen respons van gefractioneerde ALA-PDT.

Het gebruik van MAL-PDT in de behandeling van (niet-melanoom) huidkanker en acne vulgaris wordt beschreven in de **hoofdstuk 5**. Bij de behandeling van huidkanker wordt MAL-PDT meestal uitgevoerd volgens het zgn. Metvix® protocol (enkelvoudige belichting (75 J/cm^2) drie uur na het aanbrengen van MAL, herhaald na een week). PDT kan als bijwerkingen pijn geven tijdens en na de belichting. Het mechanisme van de pijn is niet duidelijk, maar er zijn verschillende factoren bekend die de pijn beïnvloeden. In dit hoofdstuk worden op basis van gepubliceerde studies enkele factoren besproken, die aangepast zouden kunnen worden met behoud van therapeutisch effect.

In een gerandomiseerde studie, beschreven in **hoofdstuk 6,** hebben we de klinische respons en het cosmetische resultaat van MAL-PDT volgens het Metvix® protocol vergeleken met chirurgische verwijdering bij de behandeling van de ziekte van Bowen. Na 18 maanden was het behandelresultaat significant beter ($P=0.018$, Fisher's exact test) voor de chirurgische verwijdering (96,0%) in vergelijking met MAL-PDT (69,6 %). Een diameter van 20 mm of groter verhoogde de kans op het terugkeren van de laesie aanzienlijk in de MAL-PDT groep. Dit verschil was net niet statistisch significant. Het cosmetisch resultaat was na 12 maanden beter voor de MAL-PDT groep ten opzichte van chirurgische verwijdering. Dit verschil was niet significant.

In een literatuurstudie, beschreven in **hoofdstuk 7,** zijn de risicofactoren voor het ontwikkelen van huidkanker binnen de verschillende subtypen van oculocutaan albinisme onderzocht**.** Door de afwezigheid of verminderde aanmaak van pigment hebben albino's een groot risico op het ontwikkelen van UV-geïnduceerde huidkanker. Algemeen wordt aangenomen dat de kans op huidkanker het grootst is in het subtype met complete afwezigheid van pigment. Wij vermoeden evenwel het tegenovergestelde. Bij verminderde aanmaak wordt er met name feomelanine pigment gemaakt. Dit is het pigment dat ook bij 'roodharigen' veel aanwezig is. Feomelanine versterkt het activeren van zuurstof. Het geactiveerde zuurstof kan vervolgens het DNA beschadigen waardoor er eerder huidkanker kan ontstaan.

In **hoofdstuk 8** worden de verscheidene stappen en hindernissen van translationele geneeskunde belicht aan de hand van onze studies betreffende gefractioneerde ALA-PDT. Translationeel onderzoek is op te splitsen in twee fasen: translatie 1 (T1), waarin kennis van ziektemechanismen wordt omgezet naar nieuwe methoden voor diagnose, therapie en /of preventie, de 'bench to bedside' ("van experiment tot patiënt") component, en translatie 2 (T2) waarin klinische studie vertaald wordt naar de dagelijkse praktijk. T1 is afgerond: preklinisch en klinisch onderzoek hebben aangetoond dat gefractioneerde ALA-PDT beter werkt dan ALA-PDT met enkelvoudige belichting. Op de afdeling dermatologie van het Erasmus MC passen we gefractioneerde PDT dagelijks toe in de behandeling van oppervlakkige niet-melanoom huidkanker. De implementatie van gefractioneerde ALA-PDT in de algemene gezondheidzorg (T2) verloopt langzaam. Factoren die de implementatie zouden kunnen beïnvloeden worden besproken.

In **hoofdstuk 9** worden de resultaten van dit proefschrift besproken in het licht van de huidige opvattingen, zoals beschreven in de literatuur, en worden toekomstige perspectieven genoemd.

CURRICULUM VITAE

Hanke Cornelia de Vijlder werd 22 oktober 1973 geboren te Castricum. In 1992 behaalde zij het VWO diploma aan het sint Nicolaas lyceum in Amsterdam. Hetzelfde jaar startte zij de studie scheikunde aan de Universiteit van Utrecht en ging in Utrecht wonen. De propedeuse werd cum laude afgelegd. Door het vak biochemie ontstond haar interesse voor de geneeskunde. Naast scheikunde startte zij in 1995 ook met de studie geneeskunde aan de Universiteit van Utrecht. In 1996 deed zij voor beide studies een stage biochemie in Madrid, Spanje. Het afstudeeronderzoek van de studie scheikunde met als onderwerp de liposomale formulering van cisplatina heeft zij op de afdeling biochemie der biomembranen en lipiden in 1999 gedaan, onder leiding van professor Dr. Ben de Kruijff. Dit onderzoek werd beloond met de “National Organon Young Research Talent Award” in 1999 en een publicatie in Nature Medicine. Het doctoraalexamen scheikunde werd aansluitend met het predicaat ‘met eer’ afgelegd. Het doctoraalexamen geneeskunde volgde het jaar daarop. In de wachttijd tot de coschappen heeft zij een half jaar als biochemicus gewerkt bij de Stichting Nederlands Instituut voor Pigmentstoornissen te Amsterdam en de afdeling immunologie van het Leids Universitair Medisch Centrum te Leiden. Daar deed zij wetenschappelijk onderzoek naar de pathogenese van vitiligo. Van 2001 tot en met 2003 volgde zij haar coschappen grotendeels in de regio Utrecht, de coschappen KNO-heelkunde en oogheelkunde in Cochabamba, Bolivia en het coschap dermatologie in Sydney, Australië. Daarna heeft ze gedurende 9 maanden gewerkt als ANIOS interne geneeskunde in het Antonius Ziekenhuis in Nieuwegein en 9 maanden als ANIOS chirurgie/SEH in het Mesos Medisch Centrum te Utrecht. In december 2004 startte zij met de opleiding tot dermatoloog in het Erasmus Medisch Centrum te Rotterdam onder begeleiding van professor Dr. H.A.M. Neumann en verhuisde zij naar Rotterdam. Onder supervisie van Mr. Dr. E.R.M de Haas en Dr. D.J. Robinson begon zij in 2007 haar wetenschappelijk onderzoek op gebied van de fotodynamische therapie. In maart 2010 heeft zij de opleiding dermatologie afgerond en is vervolgens gaan werken in het Dermatologisch Centrum Isala te Zwolle. Zij is in 2011 toegetreden tot de maatschap Zwolse dermatologen.

Hanke is getrouwd met Marcus Rietveld en samen hebben zij een zoon Joris (5 jaar) en twee dochters Carice en Marlijn (2 jaar). Zij wonen sinds 2010 in Zwolle.

LIST OF PUBLICATIONS

1. Burger KN, Staffhorst RW, de Vijlder HC, Velinova MJ, Bomans PH, Frederik PM, de Kruijff B. Nanocapsules: lipid-coated aggregates of cisplatin with high cytotoxicity. *Nature Med* 2002;**8**:81-84.

2. De Vijlder HC, Westerhof W, Schreuder GM, de Lange P, Claas FH. Difference in pathogenesis between vitiligo vulgaris and halo nevi associated with vitiligo is supported by an HLA association study. *Pigment Cell Res* 2004;**17**:270-274.

3. De Vijlder HC, de Haas ERM, Robinson DJ, Neumann HAM. Optimalisering van fotodynamische therapie met 5-ALA. *Ned T Dermatol en Venereol* 2006;**16**:22-25.

4. De Vijlder HC, Ter Borg EJ. A patient with acute renal failure. Scleroderma crisis. *Neth J Med* 2007;**65**:360-361.

5. De Haas ER, de Vijlder HC, van Reesema WS, van Everdingen JJ, Neumann HA. Quality of clinical practice guidelines in dermatological oncology. *J Eur Acad Derm Venereol* 2007;**21**:1193-1198.

6. Dih GF, de Vijlder HC, Noordhoek Hegt V, van Praag MC. Koebner-fenomeen door zonneverbranding bij psoriasis. *Ned T Dermatol Venereol* 2007;**6**:203-205.

7. De Haas ER, de Vijlder HC, Sterenborg HJ, Neumann HA, Robinson DJ. Fractionated aminolevulinic acid-photodynamic therapy provides additional evidence for the use of PDT for non-melanoma skin cancer. *J Eur Acad Derm Venereol.* 2008;**22**:426-430.

8. De Vijlder HC. Pathofysiologie van Jeuk, in Thio HB (Editor) Zakboek: Jeuk. Uitgever:Reed Business, 2008.

9. De Vijlder HC, Noordhoek Hegt V, de Man P, van Praag MC. Een cutane infectie met Mycobacterium marinum, succesvol behandeld met claritromycine. *Ned T Dermatol Venereol* 2008;6.

11. De Vijlder HC, de Haas ER, Lugtenburg P, Koljenovic S. Een patiënt met rode bulten: panniculitisachtig T-cellymfoom. *Ned T Dermatol Venereol* 2008;11.

10. De Leeuw J, de Vijlder HC, Bjerring P, Neumann HA. Liposomes in dermatology today. *J Eur Acad Derm Venereol* 2009;**23**:505-516.

11. De Vijlder HC, Middelburg TA, De Bruijn HS, Neumann HAM, Sterenborg HJCM, Robinson DJ, De Haas ERM. Optimizing ALA-PDT in the management of non-melanoma skin cancer by fractionated illumination. *G Ital Dermatol Venereol* 2009;**144**:433-439.

12. De Vijlder HC, Kemperman PMJH, Thio HB. Met de lippen verzegeld: cheilitis artefacta. *Ned T Dermatol Venereol* 2009;**11**:537-538.

13. De Vijlder HC, de Leeuw J, de Haas ERM, Neumann HAM. Licht in de behandeling van acne vulgaris. *Ned T Dermatol Venereol* 2010;4

14. De Vijlder HC, Neumann HAM. How we perform photodynamic therapy: MAL in clinical practice, in: Photodynamic Therapy in dermatology. Gold M.H. (Editor) Springer Science, 2011:pp 173-180.

15. De Vijlder HC, Sterenborg HJCM, Neumann HAM, Robinson DJ, de Haas ERM. Light fractionated significantly improves the response of superficial basal cell carcinoma to ALA-PDT: 5 year follow-up of a randomized prospective trial. *Acta Derm Venereol.* 2012;**92**:641-647.

16. De Vijlder HC , De Vijlder JJM. Genetics of human skin pigment variation, in: Hamerlinck FFV, Lambert JRMG, Neumann HAM. (Editors) *Textbook of Ethnic Dermatology,* DCHG Medical Communication, 2012:pp.52-60.

17. De Vijlder HC , De Vijlder JJM , Neumann HAM. Oculocutaneous albinism and skin cancer risk. *J Eur Acad Derm Venereol.* 2012;doi:10.1111/j.1468-3083.

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LIST OF ABBREVIATIONS:

AK	actinic keratosis
ALA	5-aminolaevulinic acid (4-oxo, 5-amino pentanoic acid)
BCC	basal cell carcinoma
BD	Bowen's disease
CD31	endothelial cell marker
CR	complete response
DHI	5,6 dihydroxy-indol
DHICA	5,6 dihydroxy-indol carboxylic acid
FU	follow-up
HAL	hexyl aminolaevulinate
ICA	intensity correlation analysis
ICQ	intensity correlation quotient
Jcm^{-2}	$Joule/cm^2$ (fluence)
LED	light emitting diode
MAL	methyl aminolaevulinate
$mWcm^{-2}$	$mWatt/cm^2 = mJoule/s.cm^2$ (fluence rate)
NMSC	non melanoma skin cancer
OCA	oculocutaneous albinism
p53	tumour suppressor protein 53
PBS	phosphate buffered salt
PDM	Product of Differences from the Mean
PDT	photodynamic therapy
PpIX	protoporphyrin IX
PS	photosensitiser
R	recurrences
RCT	randomized clinical trial
RhoA	Ras homolog gene family, member A
ROI	region of interest
ROS	reactive oxygen species
sBCC	superficial basal cell carcinoma,
SCC	squamous cell carcinoma
SNP	single nucleotide polymorphism
UV	ultraviolet light
UVA	UV: 400-320 nm
UVB	UV: 320-290 nm
UVC	UV: 290-200 nm