PHOTODYNAMIC THERAPY AND FLUORESCENCE DIAGNOSIS OF NON- MELANOMA SKIN CANCER

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LIST OF ABBREVIATIONS

ALA: 5-aminolevulinic acid / DALA: delta-aminolevulinic acid

AK: actinic keratosis

BCC: basal cell carcinoma/ sBCC: superficial basal cell carcinoma

BD: Bowen disease

COX2: cyclooxygenase 2

FD: fluorescence diagnosis / PDD: photodynamic diagnosis

5-FU: 5-fluorouracil

LED: light emitting diode

MAL: methylaminolevulinate

MMS: Mohs micrographic surgery

NMSC: non melanoma skin cancer

NRS: numeric rating scale

PDT: photodynamic therapy

PTCH: patched homologue

PPIX: protoporphyrin IX

RCM: reflectance confocal microscopy

ROS: reactive oxygen species

SCC: squamous cell carcinoma

UV: ultraviolet

1. INTRODUCTION

1.1. Non-melanoma skin cancer

1.1.1. Types of non-melanoma skin cancer

Epithelial skin cancers, also called non-melanoma skin cancers (NMSCs), are the most commonly occurring skin tumors, primarily affecting the Caucasian race, especially people with Fitzpatrick I or II skin type. Defective DNA repair mechanisms (such as in xeroderma pigmentosum) or mutations in cancer susceptibility genes (such as a mutated patched gene in Gorlin's syndrome) may lead to skin cancer even in people with darker skin types [1]. Immunosuppressed individuals, such as organ transplant recipients exposed to immunosuppressant medication over years, also have a tendency to develop skin cancers with far greater risk than the general population. The main factors leading to the development of skin cancer are ultraviolet radiation (solar UV radiation or phototherapy), ionising radiation, immunosuppression and other carcinogens, like human papilloma viruses, tar and polycyclic aromatic hydrocarbons. Carcinogenesis is a multi-step genetic process where mutations occur in genes which suppress cancer cells. Thus they become tumor promoter genes leading to uncontrolled cell proliferation and lack of differentiation. [1].

Basal cell carcinoma (BCC) is the most common human malignancy. BCC is a slowly growing, locally aggressive tumor histologically characterized by basal epithelial tumor cells. Squamous cell carcinomas (SCC) include SCC in situ (actinic keratosis, cutaneous horn, Bowen's disease (BD), erythroplasia of Queyrat), verrucous carcinoma, papillomatosis cutis carcinoides, keratoacanthoma and invasive SCC.

The pathogenesis of BCC is different from that of SCC, as it is a consequence of defects in the patched homologue (PTCH) gene in a genetic disorder (Gorlin's syndrome) or acquired usually through intermittent sun exposure. The mutated cell is activated decades later by deficient immune surveillance. Defects in DNA repair also promote BCC development. DNA repair also declines with age. Frequently a family history of BCC is obtained, so some genetic predisposition seems relevant even in sporadic basal cell carcinomas. BCCs may be multifocal [1]. Basal cell carcinoma is divided into subtypes with different clinical behavior and different aetiology. Nodular BCC is the most common (50-80 %) subtype of basal cell carcinoma followed by superficial BCC (15-25%) and the less common morpheic subtype [2].

Actinic keratoses (AKs) first present on areas of maximum ultraviolet light (UV) exposure and along with solar elastosis are the first objective evidence of cellular dysplasia. Actinic keratoses, histologically are seen as carcinoma in situ, partial thickness dysplasia, usually in the lower third of the epidermis. Progression to full thickness dysplasia may occur. It is estimated that one in 50 AKs progress to SCC. AKs are not recorded in any national cancer registries and many lesions are treated topically, so the true incidence is not known. Multifocal squamous cell carcinoma is frequently associated with the presence of human papilloma virus (HPV) and is especially common in immunosuppressed individuals. SCC is directly related to the total UV radiation dose. Eumelanin (black melanin) has the ability to absorb UV radiation. Pheomelanine (red-yellow melanine) found in skin type I and II in relatively greater proportion appears to act as a photosensitizer, generating free oxygen radicals and augmenting the effects of UV exposure. By the time AKs are present, the skin has accumulated a great amount of subclinical DNA damage, such as patches of mutated p53. The presence of actinic keratoses thus may be regarded as a major warning sign that subclinical cellular damage has been accumulated. The subclinical field changes mean the widespread presence of DNA-damaged cells which are called “field cancerization” [1].

Bowen’s disease is most common in older adults. It is a SCC in situ. The presence of multiple BDs suggests exposure to carcinogens like HPV, arsenic exposure, ionizing radiation or extended sunlight.

Genetic changes in the skin due to UV radiation are widely examined. There is a broad interindividual variation in DNA photodamage and its repair. Moreover, the differences between individuals are multiple, resulting in 1000-fold differences in sensitivity in the population, which explains the differences in the risk of skin cancer [3]. UV signature mutations in TP53 and PTCH tumor suppressor genes are responsible for non-melanoma skin cancer [4]. After UVB exposition TP53-mutated clones arise in normal human and murine epidermis and in addition to being a tumorigenic mutagen, sunlight functions as a tumor promoter [4;5]. Mutated cells must expand into a clone before becoming significant for carcinogenesis [6]. Acquiring a second mutation enables cells to proliferate autonomously. Clones of keratinocytes mutated in the p53 tumor suppressor gene grow only during chronic UVB exposure. Clonal expansion was shown to be not triggered by a proliferative mutation, but driven by continuous UVB radiation [6].

Besides the mechanical protection of stratum corneum and DNA repair, there is a third defense against UVB which prevents cells with DNA photoproducts from becoming precancerous mutant cells: apoptosis of ultraviolet-damaged keratinocytes ("sunburn cells")

[7;8]. Nucleotide excision repair genes, such as Xpa and Csb are important in apoptosis of sunburn cells [7].

The PTCH tumour suppressor gene is involved in the development of nearly all BCCs and a fraction of SCCs [9]. The single nucleotide polymorphism in codon 1315 of the human PTCH gene may play a role in increased risk for NMSC [9].

1.1.2. Epidemiology

Ninety percent of all skin cancers in the populations of Europe and North America are non-melanoma skin cancers, predominantly BCC and SCC. Incidence has increased during the past decades, but data collection is incomplete in many countries because NMSC is not routinely registered. The occurrence of NMSC increases with age, and men generally have higher incidence rates than women. Mortality from NMSC is generally very low, thus prognosis is relatively good. Mortality from SCC is approximately 12 times higher than from BCC. NMSC patients have a higher risk of a second skin cancer. Prognostic factors for NMSC include type of tumor, differentiation, localization, tumor size, invasion, age, gender, patient's immune status, and co-morbidity [2].

BCC is approximately two to four times more common than SCC. The geographic distribution of BCC and SCC varies with latitudes due to the impact of sunlight exposure on skin. A steady increase in NMSC has been observed over the last four decades among Caucasians [2]. BCC is most frequently localized around the head and neck in both genders. Among younger age groups, the proportion of BCCs in the head and neck region has decreased over the last 30 years in comparison with that of trunk, arms and legs. For both genders, the most common area of the body for SCC is the head and neck region, but in women only approximately 46% of lesions are located on the head and neck region, while 65% of lesions in men are present in these areas. The second most common region is the upper limbs, especially the dorsum of the hands. Since the incidence and prevalence of NMSC is high and there is an increased risk of de novo occurrences of NMSC, these tumors have an enormous impact on the economy of many countries. Although mortality is low, disability and disfigurement may result from these cancers [2].

1.1.3. Treatment possibilities

The gold standard in the treatment of non-melanoma skin cancer is surgical excision, but several alternatives exist, especially for superficial lesions. Considering the potential presence of subclinical lesions, just treating visible lesions does not solve the overall carcinogenic risk. Treatment possibilities which target DNA-damaged cells give a more forward-looking approach to treating sun damaged skin and prevent tumor progression. Over the past few decades treatments, both systemic and topical, have been developed to treat early sun damage and field cancerization. Topical skin cancer treatment includes 5- fluorouracil (5-FU), imiquimod, diclofenac, 5-aminolevulinic acid- (ALA-) and methylaminolevulinate- (MAL-) photodynamic therapy (PDT), intralesional interferon (IFN), intra- or perilesional interleukin-2 (IL-2). Systemic treatment includes 5-FU, isotretinoin, acitretin and systemic PDT.

Systemic and topical 5-FU generally leads to selective destruction of DNA-damaged cells with usually increased turnover and metabolism [1]. Imiquimod is an immune response modifier with antiviral and antitumoral effects, which acts through the Toll like receptor 7. Multiple clinical trials have demonstrated the efficiency of imiquimod in AK and BCC. Local inflammation is associated with this treatment, which leads to cell destruction [1]. Intralesional IFN-α2a and IFN-α2b have been shown to be an effective way to treat BCC through the expression of Fas (a component of pro-apoptotic events), downregulation of immunosuppressive IL-10 and anti-angiogenic effect [1].

Most BCCs contain genetic alterations in the hedgehog signaling pathway that result in pathway up-regulation and lead to basal-cell proliferation. Most commonly, this causes loss of function of patched homologue 1 (PTCH1), which normally acts to inhibit the signaling activity of smoothened homologue (SMO), a seven-transmembrane protein [10]. Vismodegib, a small-molecule inhibitor of the hedgehog pathway, was approved in January 2012 for the treatment of locally advanced and metastatic basal-cell carcinomas [11]. Vismodegib has had remarkable effects, particularly in patients with Gorlin's syndrome. This therapy is tailor-made for patients with basal-cell nevus syndrome. However, the side effects are considerable and frequent, resulting in high rates of drug discontinuation, and these rates will probably be even higher in clinical practice [12].

SCC and precursor lesions express more cyclooxygenase 2 (COX2), so blockade of COX2 leads to regression of these lesions [1]. Topical diclofenac is an easy and simple method

for the treatment of AKs and subclinical lesions. The mechanism of action in topical diclofenac treatment of AK is not clearly understood, although inhibition of COX enzymes is suggested by some researchers. Recurrence of AKs is frequent after diclofenac therapy [1]. Cryotherapy of individual lesions is also a simple and rapid way to treat superficial BCC, BD or AKs, but it may lead to scar formation, which impairs clinical judgement of a possible tumor recurrence and results in unpleasant cosmetic outcome. Systemic retinoids proved to be useful in skin cancer prevention in immunosuppressed or genetically predisposed patients. In addition photoprotection, usage of sunscreens and sun avoidance are very important to prevent skin tumours [2].

An optimal cosmetic result is often given a higher priority than total removal due to the slow growth and low metastatic rate of these tumors.

1.2. Photodynamic therapy and fluorescence diagnosis

PDT has recently become accepted for the treatment of non-melanoma skin cancer.

1.2.1. History of Photodynamic therapy

The foundation of PDT was laid by Oscar Raab over 100 years ago, when in 1900 he discovered that the illumination of Infusoria cell cultures in the presence of acridine would result in apoptosis [13]. In 1905 Hermann von Tappeiner and Jesionek used phototherapy after the application of eosin to treat non-melanoma skin cancer [14;15]. Further on possibilities inherent to photodynamic therapy were recognized again and again. In 1960 Lipson and Baldes observed that after topical administration of porphyrin derivates fluorescence could be detected in the area of skin tumor in ultraviolet light. Preparation of the so called hematoporphyrin derivate is due to Schwartz. Dougherty processed the complete method of „photoradiotherapy" of malignant tumors in 1987 [13;15]. Formerly systemically applied photosensitizers were used [16] but in the last decade, topically administered non-toxic and non-carcinogenic photosensitizers, like 5-aminolevulinic acid or its methyl esther, methyl-aminolevulinate have become popular [17]. Topical photodynamic therapy with ALA was first described by Kennedy et al in 1990 [18].

1.2.2. Biophysical bases

The essence of PDT is that the introduction of an exogenous photosensitizer into the skin cells results in the accumulation of protoporphyrin IX (PPIX) in rapidly proliferating cells. During therapy, the skin surface is illuminated at an appropriate wavelength, which leads to the excitation of PPIX. As the PPIX returns to the basic energetic state, reactive oxygen radicals (reactive oxygen species, ROS) are generated, and these induce the apoptosis of tumor cells [19]. The wavelength of the light used for illumination must be appropriate for the peaks in the absorption spectrum of PPIX. The often used wavelength of 630 nm does not match the largest peak in the absorption spectrum, but penetrates the skin better than green or blue light with lower wavelengths (although green or blue light excites PPIX more intensely) [13;20].

1.2.3. Photosensitizers

Topically applied photosensitizers, like 5- or delta-aminolevulinic acid (ALA) or its methyl ester the methyl-aminolevulinate (MAL), by themselves do not cause sensitivity to light. However, they metabolize in the cells, thus resulting in the formation of photosensitising protoporphyrin IX in the process of heme synthesis.

The accumulation of photosensitising porphyrins also depends on the availability and the quantity of Fe 2+ ions, since in their presence PPIX develops into heme. The right concentration of photosensitizers within the treatable cells depends on the transport of photosensitizer molecules through the cell membrane.

While ALA enters cells mainly through active transport, MAL, possessing lipophilic properties, can also enter cells through passive transport [21]. In the case of MAL the difference between tumorous cells and healthy cells are also more explicit. After entering the cell, however, MAL also metabolizes into ALA, and henceforth the same biochemical processes take place in the case of both photosensitising agents. With epithelial tumors thicker than 2-3 mm the effectiveness of the PDT is limited by low penetration of the photosensitising agent and light [22]. In the last 15 years the development of the so called "second generation" photosensitising agents [phtalocyanin, naphtalocyanin, chlorin, Verteporfin (Visudine®, Novartis), Temoporfin (Foscan®, Biolitech Pharma), indocyanine green] have started. These are synthetically made constant structured prophyrin derivates; their maximum absorption

falls within the red, almost infrared range (660–850 nm). Their advantage is a deep, up to 20 mm deep penetration, their disadvantage is the solely intravenous applicability since these molecules do not penetrate through the skin [23]. Their halflife measured in the plasma is short thus not causing prolonged photosensitivity.

1.2.4. Light sources

In choosing the light source used during PDT two important factors must be kept in mind: 1. the applied light must reach the layer of skin which is to be treated, 2. its wavelength must line up with the peaks of the absorption spectrum of PPIX. It is a generally accepted rule that the penetration of light into the skin is directly proportional to the wavelength - blue light absorption is the most superficial, red and infrared penetrates most deeply. On the absorption curve of the PPIX the highest peak is at 405 nm, this wavelength corresponds to blue light emitting light sources. Even though this wavelength range efficiently excites the PPIX, its disadvantage is the only minimal penetration into skin. Further absorption peaks can be found at 510, 545, 580, 630 nm. On the absorption curve of the PPIX the highest peak does not belong to the 630-nm wavelength light – the most commonly used wavelength in the oncodermatological practice. At the same time red light penetrates deeper than the shorter wavelength blue or green light [13;20]. The broad-spectrum, high-pressure fluorescent and halogen lamps are cheap and are able to emit large amounts of energy. LED (light emitting diode) lamps emit a narrow spectrum of light, their advantage is that when built into panels they make constant energy density irradiation possible on larger areas of skin. The advantage of a laser is that its wavelength is closely in line with its photosensitizer absorption maximum, but it is expensive, not portable and generally the high energy of a laser is not required. The use of laser light in PDT may be considered primarily in the photodynamic treatment of internal organs. Intense pulsed light (IPL) is mainly used for PDT-photorejuvenation [24].

1.2.5. Practice of topical PDT

Photodynamic therapy is a non-invasive procedure. Before treatment, the skin tumor is subjected to chemical and mechanical (Volkmann’s spoon) keratolysis. Then the photosensitizer (20% ALA or 16% MAL) ointment is applied for 3 or 4 hours in an occlusive, light-reflecting dressing. The photosensitizer is applied approximately 0.1 cm thick with a spatula, with a 1-cm overlap onto the surrounding tissue area. After photosensitizing, the

dressing is removed, together with the residual ointment. With the monochromatic diode lamp (Aktilite®, PhotoCure ASA, Oslo, Norway) situated 8 cm from the skin surface, illumination is performed with 630 nm visible red light at a dose of 37 J/cm^2. The patients are recommended to use 50 SPF sunscreen for 1 week after the treatment. At a follow-up visit physical examination is performed to evaluate the necessity of further treatment. PDT is an indefinitely repeatable method without influence to other subsequent treatments.

1.2.6. Side effects and limiting factors

Limiting factors of PDT include thick tumours (> 2 mm), and the pigment content of tumours (melanin pigment absorbs light). PDT is contraindicated in morpheic BCC. Among the most common side-effects of PDT are erythema, crusting, serous discharge, oedema, and sterile pustule formation, developing several hours after the treatment. Therapy-related pain is the most frequent side-effect, which often limits the duration of treatment, and the efficiency. Patients usually report a cumulative burning sensation during illumination that becomes intense within a few minutes after the start of the procedure. In some cases, the pain may become so severe that the illumination must be stopped prematurely, with the result that the applied light dose, and the PPIX formation are insufficient, and the therapeutic result is inadequate. The unpleasant experience associated with the treatment does not promote patient compliance. Pain and pain relief during PDT is therefore a very substantial factor.

1.2.7. Photodynamic therapy of non-melanoma skin cancers

Topical PDT is a highly effective mode of treatment in AK and superficial BCC. Prospective, randomized studies have proven that in cases of AK response rate for MAL-PDT was similar or better than cryotherapy (complete remission 69-89% vs 67-75%, respectively) [25-28], however its cosmetic outcome is far more superior [25;28]. In case of superficial BCC recurrence rates with ALA- or MAL-PDT are also similar with cryotherapy [29;30]. In large and multiple AKs and superficial BCCs, PDT is more suitable than other forms of treatment [31]. In the treatment of AK, PDT has been demonstrated to be more effective (80% vs. 69%, respectively), and to deliver an excellent cosmetic result and is much more tolerable than 5-fluorouracil (5-FU) [25;32]. In case of nodular BCC, the efficiencies of PDT and surgical excision do not differ notably (complete remission 91% vs 98%, respectively), but the 1-year and 5-year recurrence rates were slightly higher in the PDT-treated group [33]. Topical PDT in

BD is at least as effective as cryotherapy or 5-FU, but has fewer adverse effects [31]. Accordingly, PDT is currently regarded as first line treatment in case of AK, superficial BCC and BD [31]. In non-melanoma skin cancers, MAL and ALA are equally effective as photosensitizers in PDT [34;35]. PDT may also be utilized to prevent certain non-melanoma skin cancers in organ transplant and other immunosuppressed patients [31].

1.2.8. Fluorescence diagnosis

PPIX accumulated in epithelial cells may be detected with the help of ultraviolet light by its fluorescence. On UV imaging the coral red - cyclamen fluorescence outlines the border of the damaged or neoplastic area. Thus fluorescence diagnosis (FD) allows an accurate assessment of tumor borders and may be used on one hand to perform guided biopsy, on the other hand to plan the lines of excision prior to surgical removal [36]. Furthermore, it may help at follow-up after PDT in deciding whether the repetition of the treatment is necessary when the response to therapy is difficult to judge. The fluorescence ratio between neoplastic area and surrounding tissue must be maximal. Therefore, it is of utmost importance to set the incubation time of the photosensitizer properly. At the beginning of the incubation the rapidly proliferating abnormal, photo-damaged or malignant cells with rapid metabolism take up the porphyrin precursor. After some time the visible difference between the detectable fluorescence of the neoplastic area and the surrounding healthy area is increasing. However with extended incubation time also normal cells take up the porphyrin precursor. At this moment the fluorescence ratio between tumor and intact tissue is decreasing again. The optimal fluorescence ratio is attained after about 3-14 hours of incubation [36-38]. Thus for photodynamic diagnosis (PDD) and therapy generally a 3-hour photosenzitizing period is used.

1.2.8.1. Fluorescence imaging systems

For the purpose of the routine usage of fluorescence diagnostics many imaging systems (Dyaderm, Biocam, Germany [39], Photodemarcation system 1, prototype 5, Medeikonos AB, Sweden [40]) were created, some of which are suitable for quantitative measurement of the porphyrin accumulation and can provide an accurate calculation of the fluorescence ratio of the surrounding skin and tumor. It is important that the procedure is properly standardized since the photo shooting distance and angle, the location of the specific

area and the lighting conditions all have an effect on the measured amount of PPIX [39]. The great advantage of these systems is that they are able to make simultaneous color (morphological) and fluorescence (physiological) recordings, and the two photos can also be summarized. After appropriate standardization reproducible shots can be taken before and after the treatement and also every time the patient comes for a check up. There is also a possibility for the temporal measurement of prophyrin kinetics so that optimal photosesitizing incubation time can be selected which results in the greatest difference in the porphyrin accumulation of surrounding tissue and skin tumor.

1.2.8.2. Fluorescence diagnosis, a method to simplify Mohs micrograhic surgery

Mohs micrographic surgery (MMS) is especially used for the treatment of facial basal cell carcinoma. Its essence is that the excision of the tumor is started with a 1-2 mm wide safety zone beyond the visible edges and the removed tissue sample is marked with different colors to maintain correct orientation. Before defect closure a quick histological processing and examination of the removed tissue is done. If the excision line is not tumor free that is that the removal was not done in healthy skin, additional layers of excision are performed until there are no more tumor cells in the examined tissue. Only after complete excision it is permitted to close the evolved defect. Mohs micrographic surgery has proven the most effective form of treatment for high-risk basal cell carcinoma located on the ear and the middle of the face [41]. Tumors located in these areas often threaten the integrity of eyelids, nose cartilages, lips, as their size cannot always be judged clinically. In the facial area it is a top priority to attain a proper cosmetic result. Since lobe rotation or skin graft transplantation is often needed it is of utmost importance that the excision is done within healthy tissue to avoid the need for reexcision. This is why an accurate determination of the edges of the lesion is of utmost importance. Mohs micrographic surgery increases the effectiveness of the excision in healthy tissue, however, it is time-consuming, cumbersome and expensive. Thus the need for increasing cost-effectiveness and ease of implementation arose. Previously, for this purpose PET CT, spectrophotometral intracutaneous analysis and laser Doppler velocimetry were used but all of these require a complex and specially trained staff [36]. Tierney and his colleagues detected sufficient contrast in UV light between fluorescence of the tumor and of its surrounding after a 12 to 14 hour MAL incubation. At that time they measured the base area of the tumor based on fluorescence [36]. The "Mohs surgeon" could not see the

fluorescence and the data of the tumor size. After complete removal of the tumor the researchers measured the size of the defect. The tumor size measured by fluorescence and the defect created during the (Mohs) surgical procedure were closely correlated [36]. In contrast, the size of the tumor seen during a normal physical examination was much lower [36;40]. All this proves that based on fluorescence, the extent of the tumor and its edges can be judged much more accurately. This technique reduces the number of excisions in Mohs microsurgery thus shortens the surgical time. Also other authors have judged the determination of the edge of a tumor with the help of fluorescence diagnostics. Sclerodermiform basal cell carcinomas and BCCs showing trichilemmal differentiation gave false negative results [42].

1.2.8.3. Fluorescence diagnosis to judge therapeutic result

At a follow-up visit the most accepted method to judge the effectiveness of treatment and the occurrence of recurrence was done physically (visual, both dermoscopic and tactile). Photodynamic fluorescence enabling to determine the actual size of the tumor helps in giving a more accurate assessment of the therapeutic response and recurrence. At follow-up visit a fluorescence test under UV light is performed if complete remission cannot be judged, after a three-hour MAL or ALA photosensitizer incubation. If a well-defined area with stronger fluorescence than the surrounding area is detected, the photodynamic treatment can be repeated. After a MAL photosensitization a higher contrast can be detected between the tumor and surrounding healthy tissue than when using ALA [40], therefore when using fluorescent diagnostics MAL is preferable. MAL's higher tumor selectivity has been known for quite some time [43;44].

1.2.8.4. Limiting factors of fluorescence diagnosis

The disadvantage of photodynamic diagnosis is that it does not give information about the thickness of the tumor. It is perfectly applicable for superficial basal cell carcinoma, actinic keratosis and Bowen disease. In patients with specially photodamaged skin, for instance before the excision of a basal cell carcinoma, fluorescence diagnostics is not necessarily beneficial, since the fluorescence of the basal cell carcinoma and the surrounding actinic keratoses can blend into one, making the area of the excision larger than actually needed. Yet surgical removal of AKs is not always necessary [42]. In cases of superficial BCC it is better to apply photodynamic therapy.

1.3 Diagnosis of non-melanoma skin cancers with in vivo reflectance confocal microscopy

The in vivo reflectance confocal microscopy (RCM) technique allows examination of the superficial areas of the skin, the epidermis and the upper part of the dermis with high resolution images and the recording of these images. The skin structure and various skin lesions can be examined with the confocal laser scanning microscope in vivo, "real time" mode or they can be tracked with the help of standard recordings taken at different occasions. This way we are able to get a picture of the skin which can even be similar to histomorphology without being invasive. In certain cases, biopsy can be avoided with this technique, which is particularly important when we plan for a non-invasive treatment promising a better cosmetic result than standard therapy, like in the case of the photodynamic therapy of AK, sBCC or BD. With confocal microscopy the pre-treatment analysis, diagnostics of skin lesions selected for PDT, and also tracking the changes in these lesions as a result of the treatment became possible. In addition, at the follow-up visit the effectiveness of therapy can be judged and the possible presence of subclinical residual lesions can be detected. Since the thickness of skin lesions suitable for PDT usually coincides with the domain which we can examine using a RCM device (0-200 μm), the method is suitable for examining these premalignant skin lesions and tumors before and after treatment.

Based on the literature, actinic keratosis and Bowen disease with in vivo reflectance confocal microscope can be diagnosed based upon the following criteria: in the area of stratum corneum, disruption, parakeratosis, and hyperkeratosis is characteristic. In the epithelium in the area of the stratum granulosum-spinosum architectural disarray (irregularly edged keratinocytes with irregular shape and size, which is the irregular honeycomb pattern) and cellular nuclear pleomorphism, exocytosis, spongiosis are visible. The upper dermal area is characterized by solar elastosis, increased vascularisation, and the presence of dilated blood vessels, the so-called lymphocyte rolling and inflammatory infiltration [45-48]. BCC has a typical RCM image also. The nuclei and cells elongate, so they become monomorphic and they become polarized. This appears partly as longitudinal bundle-like arrangement, partly as palisade ordering on the edge of tumor nests. The epithelial cells may become irregular. Inflammatory infiltrate and vascularity may be explicit. Lobulated tumor cell-nests can be detected in batches which depending on the pigment content may appear with extent

reflectivity (light islands of tumor cells) or may be dark (dark silhouettes). Around them slit-like, dark zone can be seen [49-51].

2. AIMS

2.1. Comparison of pain during PDT with two different photosensitizers, ALA and MAL

The aim of our study was to evaluate the degree of treatment-associated pain during PDT with two different photosensitizers, ALA and MAL, in different anatomical regions, consideration being given to differences in diagnosis, age, gender and the method of pain relief. Relatively few data are available so far concerning the level of pain during PDT when these two frequently applied topical photosensitizers are used. Pain is the most important undesired reaction of PDT. MAL and ALA are the most common photosensitizers. It is already known that ALA usage is a predictive factor for higher pain level, but the correlation between the type of photosensitizer, the localization and the diagnosis of lesion, age and gender of patient simultaneously with PDT associated pain has been poorly investigated.

2.2. Fluorescence diagnosis and in vivo confocal reflectance microscopy in skin cancer diagnostics and for assessment of the PDT-related changes and therapeutic response

Our goals were to supplement the traditional physical (visual, tactile and dermoscopic) examination of epithelial skin tumors before PDT with other non-invasive diagnostic methods like in vivo reflectance confocal microscopy and fluorescence diagnostics. We wanted to examine the potential detectability of protoporphyrin IX accumulation using confocal microscopy and to monitor the changes seen in the tissue immediately after photodynamic therapy. Furthermore we intended to investigate the effectiveness of therapy using in vivo confocal microscopy and fluorescence diagnostics.

3. PATIENTS AND METHODS

3.1. Examination of pain during PDT with two different photosensitizers, ALA and MAL

During the 4 years between December 2003 and November 2007, PDT was performed on 182 occasions to treat non-melanoma skin cancer in our Department. Eighty-seven patients were involved (32 females, 55 males, mean age=72 years, age range=43-92 years). The locations of the tumors were as follows: head and neck region: 111 (cheeks: 22, forehead: 31, temporal area: 12, nose: 18, auricular region: 12, lip: 1, scalp: 11, neck: 4), trunk: 45 (back: 29, chest: 15, abdomen: 1), and extremities: 26 (shoulders: 13, arms: 6, hands: 5, shin: 1, thigh: 1). Before treatment, patients gave their informed written consent. Punch biopsies of the lesions were made for histological confirmation of the diagnosis. PDT was performed on 80 occasions for AK, 97 occasions for BCC and 5 occasions for BD. Before treatment, the skin tumors were subjected to chemical (20% salicylic acid in vaselinum acidum boricum ointment) and mechanical (Volkmann's spoon) keratolysis. Patients were randomly assigned to receive either 20% ALA or 16% MAL ointment for 4 hours in an occlusive, light-reflecting dressing. The 20 % ALA ointment was prepared by the Pharmaceutical Department of our University: 200 mg ALA (Sigma Chemical company, US) in 1 g non-ionic hydrophilic ointment, as published previously [52]. The commercial MAL cream contained 160 mg MAL hydrochloride in 1 g cream (Metvix®, Galderma). The photosensitizer was applied approximately 0.1 cm thick with a spatula, with a 1-cm overlap onto the surrounding tissue area. ALA was used for 103 treatments (48 patients, 18 females, 30 males, age range=46-92 years, mean age=72 years) and MAL for 79 treatments (39 patients, 14 females, 25 males, age range= 43-87 years, mean age=70.4 years). 30 minutes prior to illumination, the patients were offered, and if requested, administered 500 mg paracetamol orally for pain relief. However, it is of note that currently applied oral analgesics do not affect considerably the pain scores. After 4 hours of photosensitizing, the dressing was removed, together with the residual ointment. With the monochromatic diode lamp (Aktilite®, PhotoCure ASA, Oslo, Norway) situated 8 cm from the skin surface, illumination was performed with 630-nm visible red light at a dose of 37 J/cm^2 (duration of 12 minutes). During the illumination, the skin surface was cooled periodically with wet gauze, if requested by the patient. Both groups were treated equally with regard to analgesia before and during

PDT. The patients were recommended to use 50 SPF UV filter cream after the treatment for 1 week. The degree of patient-reported pain was assessed immediately after PDT on a 0-10 numeric rating scale (NRS), which was explained to each patient before the therapy (0 meaning no pain and 10 meaning unbearable pain). NRS is an accepted method for pain measurement [53].

One and two-way ANOVA, Student's T-test and the Scheffe test (*post hoc*), were applied for statistical analysis with Statistica 8.0 software (StatSoft Inc.). Scheffe's method was used to adjust significance levels for multiple comparisons in ANOVA. Differences were considered statistically significant at $p<0.05$. Four weeks after the first treatment, a follow-up examination was performed to evaluate the necessity of further treatment. Comparison of the pre-treatment and follow-up photo documentation and the results of physical examination were used to evaluate treatment efficacy. The tumors were rated into 3 groups from the aspect of the therapeutic result: complete remission (CR), incomplete remission (IR), or no response (NR) by an independent physician who did not know whether ALA or MAL has been used for the treatment. If any residual tumor was seen at the follow-up visit, repetition of the PDT was suggested.

3.2. Examination of adjuvant methods for monitoring the efficacy of PDT

Since December 2003 we have been routinely performing photodynamic treatments of superficial basal cell carcinoma, actinic keratosis and Bowen's disease at our Department. During this time nearly 500 patients were treated this way.

In our present study 12 patients (6 men, 6 women) were enrolled with whom PDT was planned in AK, sBCC and BD indications. The mean age of patients was 68.9 (age range 45-83 years). After we had given detailed oral and written information, with the help of an Olympus E-330 digital SLR camera and Clearstone ultraviolet digital imaging analysis system, normal and then UV photos were taken of the lesion that was to be treated and confocal microscopy was performed. The PDT-treated lesions were distributed as follows: 4 actinic keratoses, 7 superficial basal cell carcinomas, 1 Bowen disease. The clinical diagnosis was confirmed by a confocal microscopic examination prior to treatment (in 7 cases a biopsy were made also before treatment), the lesions showed typical signs appropriate for the three above mentioned diagnosis on the RCM image.

3.2.1. In vivo confocal reflectance microscopy with three different wavelength lasers before and after photosensitization and immediately after photodynamic therapy

When taking confocal microscopy images we used the VivaScope® 1500 Multilaser (MAVIG GmbH, Munich, Germany). The device operates at three wavelengths: 785 nm (near infrared), 658 nm (red) and 488 nm (blue). We investigated whether there was a difference between the image made by the three wavelengths and whether the photosensitizer ALA containing cream or PDT therapy would cause any change on the RCM images. One selected actinic keratosis was recorded with all three lasers before applying the photosensitizer, immediately after removal of the cream and immediately after illumination.

3.2.2. Fluorescence diagnosis

Photodynamic therapy of superficial basal cell carcinoma, actinic keratosis and Bowen's disease are carried out routinely in our practice. After careful removal of any hyperkeratosis, for 3 hours, a 20% ALA, or a 16% MAL cream is applied on the skin surface and covered with an occlusive, non-translucent bond.

Subsequently, with the help of a Olympus E-330 digital SLR camera and Clearstone ultraviolet digital imaging analysis system, a normal then a UV photo is taken first. After the shooting, the tumor is illuminated with 630-nm visible red light at a dose of 37 J/cm^2 (duration of 12 minutes) (Aktilite®, PhotoCure, LED light source). After illumination a third UV photo was taken. Four weeks later at the follow-up visit another normal and UV photo was taken.

3.2.3. In vivo confocal reflectance microscopy before, immediately after and four weeks after PDT treatment

For taking the confocal microscopy images the VivaScope® 1500 Multilaser (MAVIG GmbH, Munich, Germany) was used. The device operates at three wavelengths: 785 nm (near infrared), 658 nm (red) and 488 nm (blue). First, we took a dermoscopic picture with the help of the camera of the selected lesion. In each case, we set the baseline "zero" height based on the image of the machine's retaining ring. After this we set the border of stratum granulosum-

spinosum of the surface epithelium. This level is typically located 20-25 microns lower than the baseline. From this point on we took 8x8 mm pictures using the maximum field-vision of the machine. We took images in three layers every 25 microns. Then, according to our clinical practice we applied a 20% ALA photosensitizer ointment and an occlusive dressing to the skin to be treated for 3 hours. After the photosensitizer incubation time had passed we took another UV photo. On the digital UV photo, the strong fluorescence of the tissues accumulating PPIX was clearly visible. After the shooting, for 12 minutes, at a dose of 37 J/cm2, with a 630 nm light (Aktilite®, PhotoCure ASA, Oslo, Norway, LED light source) the tumor was illuminated. At this time the detectable fluorescence was much less in the UV light because PPIX degraded. After irradiation once again a confocal microscopy image recording was done in order to examine the immediate changes caused by the treatment. After 4 weeks we called our patients back for a follow-up visit. Then, in addition to physical examination, once again normal and UV photos were taken and in vivo confocal microscopy examination was performed.

4. RESULTS

4.1. Pain during photodynamic therapy

4.1.1 Pain associated with photodynamic therapy in different anatomical regions

Immediately after PDT, the patients estimated the degree of pain felt during PDT on the scale 0-10; 10 denoting intolerable pain, and 0 no pain. Ten patients with a total of 24 treatments experienced intolerable pain necessitating premature disruption of the treatment. In 21 of these discontinued treatments involving 9 patients, the photosensitizer used was ALA; only 1 patient treated with MAL-PDT in 3 different anatomical regions requested premature discontinuation. As concerns the former 21 disrupted treatments, 14 of the tumours were AKs (17,5% of the total number of AKs) and 5 were BCCs (5,1% of the overall BCCs). Many of these lesions were localized on the cheeks (n=7) or forehead (n=4).

Regions on the head, and especially the cheeks, forehead and scalp proved to be the most painful areas, but the extremities were also sensitive. The differences of PDT associated pain in different anatomical regions, however, were not significant (one-way ANOVA, p=0.17 and p=0.09) ***(Fig. 1., Fig. 2.)***

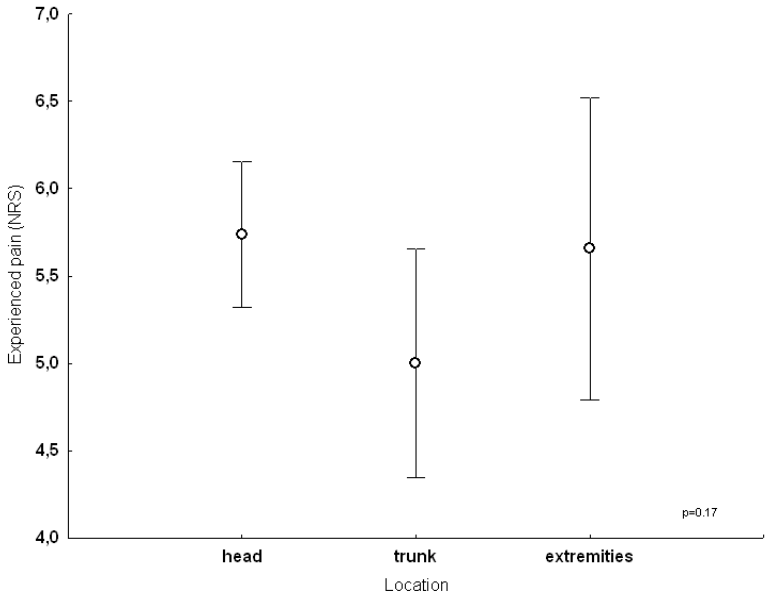

Figure 1. PDT associated pain in different anatomical regions (mean NRS values with SD bars). Although the head and the extremities were more sensitive than the trunk, the differences of PDT associated pain in different anatomical regions were not significant (one-way ANOVA, p=0.17).

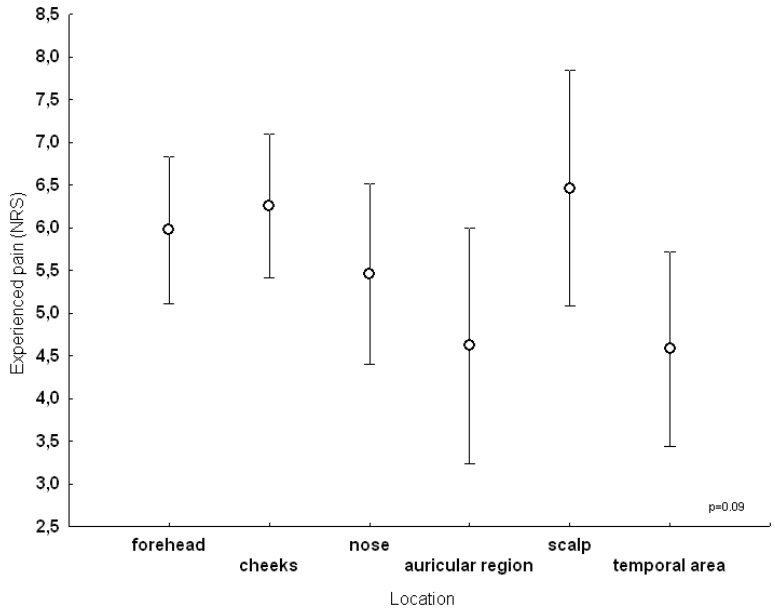

Figure 2. PDT associated pain in different regions of the head (mean NRS values with SD bars). PDT in the cheeks, forehead and scalp areas was found more painful than in the nose, auricular or temporal regions, however, the differences were statistically not significant (one-way ANOVA, p=0.09).

4.1.2 Comparison of PDT associated pain in different anatomical regions using two different photosensitizers, ALA and MAL

The levels of pain in the regions of the head, trunk and extremities during PDT were compared between the groups receiving the different photosensitizers, ALA and MAL. In the head region, MAL-PDT caused significantly less pain than did ALA-PDT (two-way ANOVA, Scheffe *post hoc* test, p1=0.00068). There was a tendency for ALA-PDT to be more painful in all examined anatomical regions, but in the regions of the trunk (p=0.062) and the extremities (p=0.19) the differences were not significant ***(Fig. 3.)***.

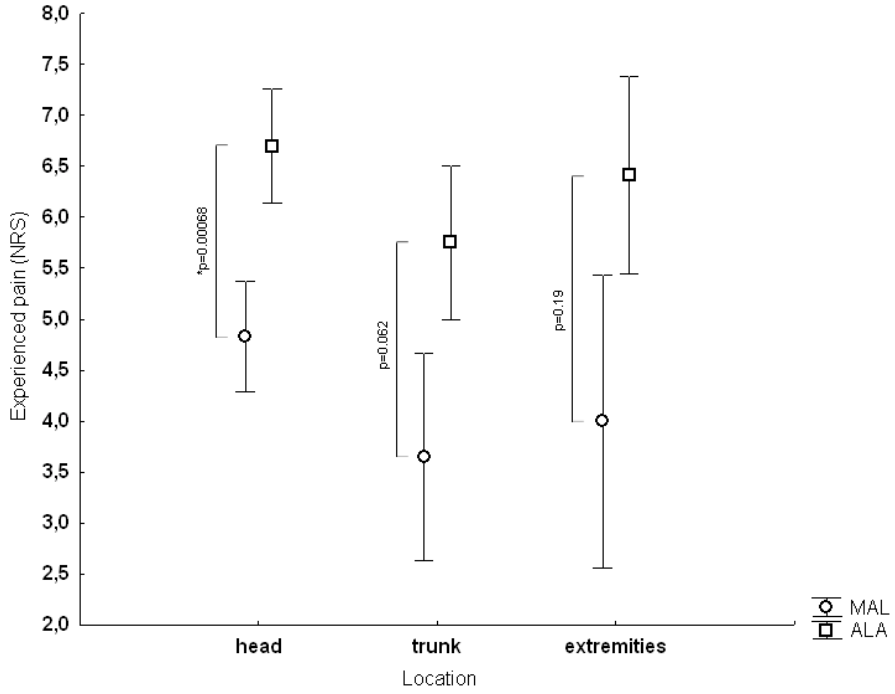

Figure 3. Comparison of MAL- and ALA-PDT associated pain in different anatomical regions (mean NRS values with SD bars). In the sensitive head region, MAL-PDT caused significantly less pain than ALA-PDT (two-way ANOVA, Scheffe *post hoc* test, p=0.00068). There was a tendency for ALA-PDT to be more painful in all examined anatomical regions, but in the extremities (p=0.19) and trunk (p=0.062) regions the differences were statistically not significant.

4.1.3 Comparison of PDT associated pain according to different types of lesions

The level of pain during the PDT of AK was significantly greater than that in the case of BCC (two-way T-test, p=0.0025) ***(Fig. 4.)***.

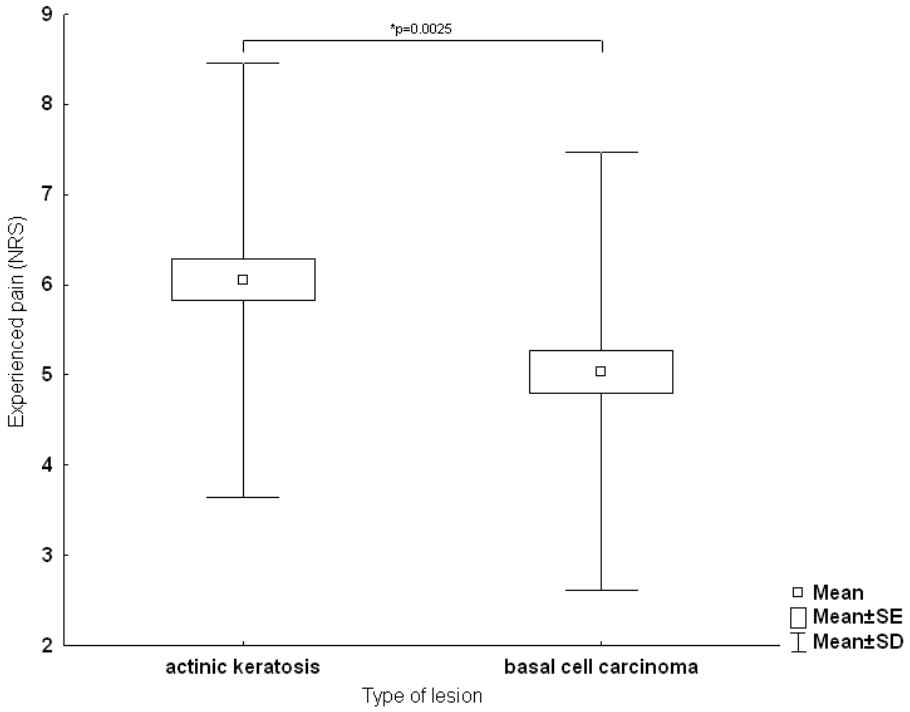

Figure 4. The amount of pain that the patients experienced according to the histologically different groups of lesions (mean NRS values with SE boxes and SD bars). The level of PDT associated pain during the treatment of actinic keratoses was significantly greater than that in the case of basal cell carcinomas (two-way T-test, p=0.0025).

Despite the difference being significant for the overall AK and BCC groups, breakdown in regard to the two photosensitizers resulted in a significant difference only in the MAL group (two-way ANOVA, p=0.0025, Scheffe *post hoc* test, p1/MAL-AK:MAL-BCC/=0.039; p2/ALA-AK:ALA-BCC/=0.63) (Fig.5.). In the BCC and AK groups, significant differences were detected between MAL and ALA (p3/MAL-AK:ALA-AK/=0.0083; p4/MAL-BCC:ALA-BCC/=0.00001) in concordance with the observation that MAL-PDT caused less pain ***(Fig.5.)***.

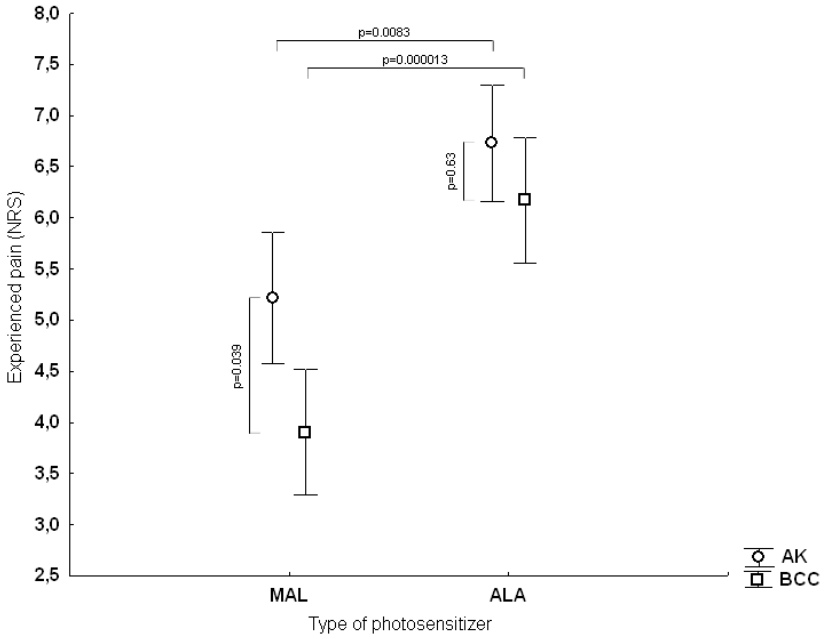

Figure 5. Comparison of MAL- and ALA-PDT associated pain in patients with actinic keratosis and basal cell carcinoma (mean NRS values with SD bars). Pain during BCC treatment was lower in both the ALA and MAL groups, however, only reached statistical significance in the MAL group (two-way ANOVA, p=0.0025, Scheffe *post hoc* test, p1/MAL-AK:MAL-BCC/=0.039; p2/ALA-AK:ALA-BCC/=0.63). MAL-PDT caused significantly less pain than ALA-PDT in both diagnosis groups (p3/MAL-AK:ALA-AK/=0.0083; p4/MAL-BCC:ALA-BCC/=0.00001).

4.1.4 PDT associated pain in different genders

There was no significant difference in the degree of pain between the genders (two-way t-test, p=0.19) ***(Fig. 6.)***.

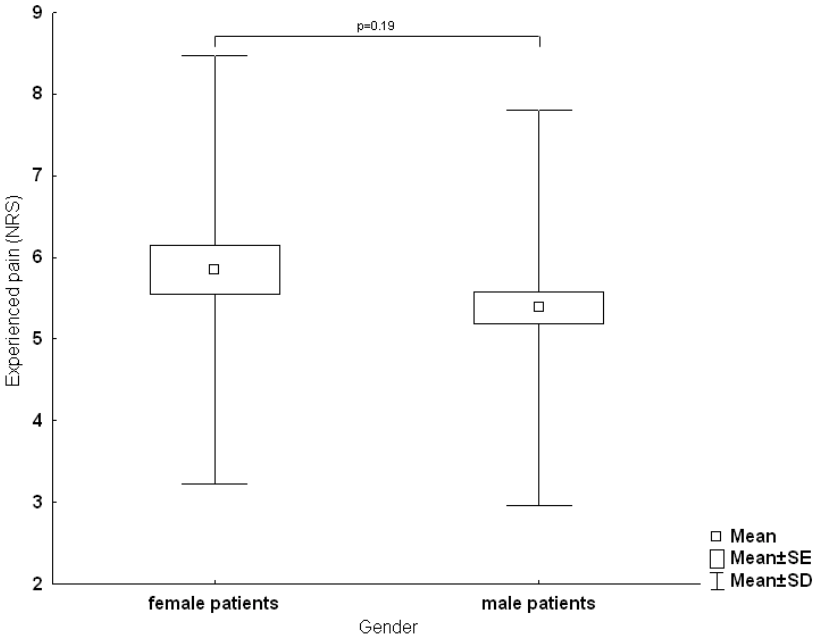

Figure 6. Comparison of PDT associated pain between female and male patients (mean NRS values with SD bars). There was no significant difference in the degree of pain between the genders (two-way t-test, p=0.19).

4.1.5 PDT associated pain according to age

Increasing age was significantly associated with more pain sensation. We assessed and compared pain in the following age groups: 40-59 years (n=10), 60-79 years (n=60), over 80 years (n=17), and found significant difference between 40-59 and over 80 years group (one-way ANOVA, p=0.008, Scheffe *post hoc* test, p1/40-59:60-79/=0.3; p2/60-79:>80/=0.057; p3/40-59:>80/=0.014) ***(Fig.7.)***.

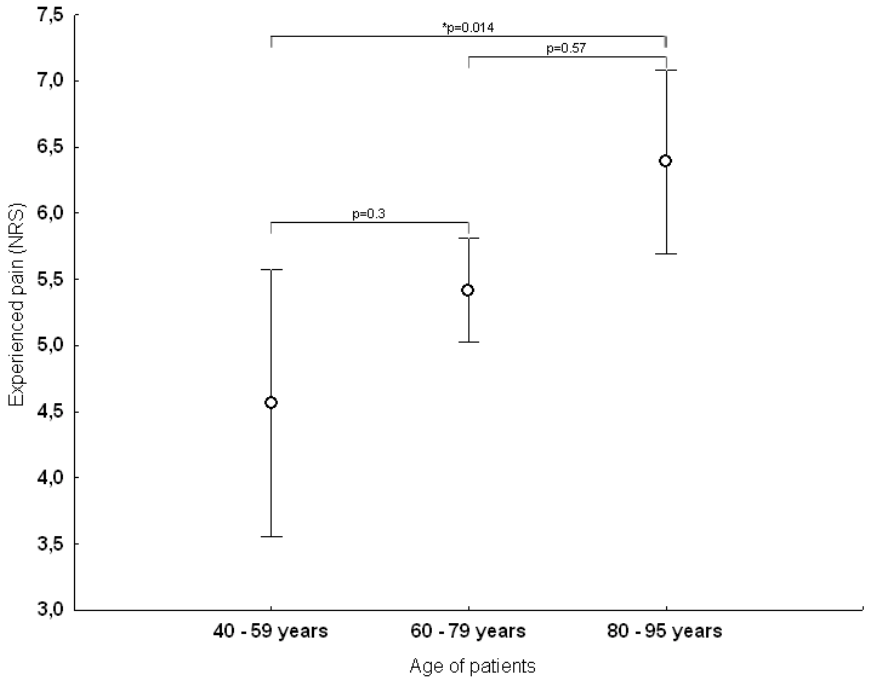

Figure 7. PDT associated pain in different age groups (mean NRS values with SD bars). Increasing age was significantly associated with more pain sensation. Pain was compared in the following age groups: 40-59 years (n=10), 60-79 years (n=60), and over 80 years (n=17). Significant difference was found between the youngest and the oldest groups (one-way ANOVA, p=0.014), while the difference between the 40-59 and 60-79 years groups, and the 60-79 and 80-95 years groups were statistically not significant (p=0.3 and p=0.057, respectively).

4.1.6 Effects of pain alleviating methods

A significant difference was not observed between the different methods of pain relief: cooling the skin with wet gauze during treatment, oral analgesia, or both (one-way ANOVA, p=0.77) ***(Fig. 8.)***.

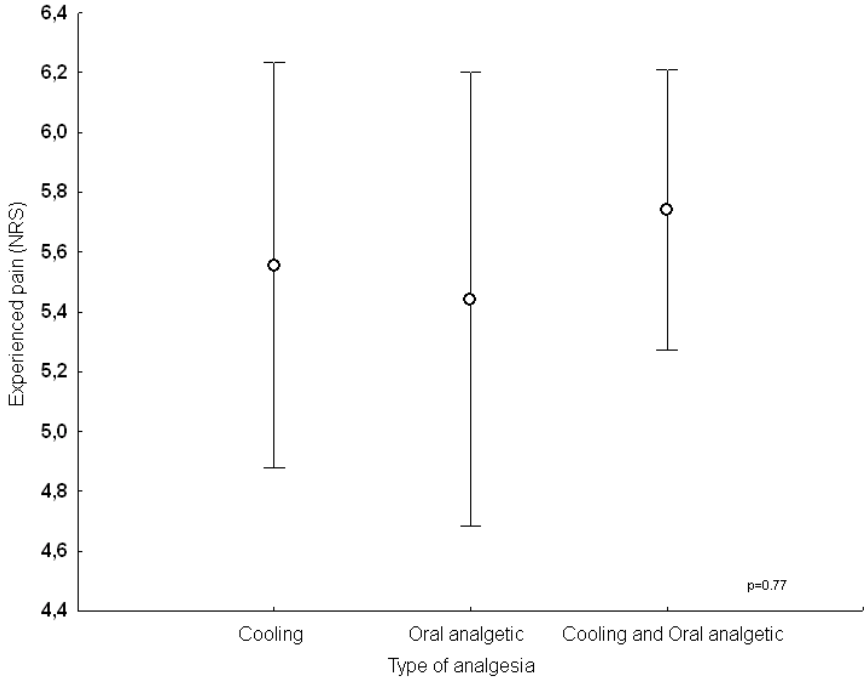

Figure 8. Effect of different methods of pain relief on PDT induced pain (mean NRS values with SD bars). **S**ignificant difference was not observed between the different methods of pain relief: cooling the skin with wet gauze during treatment, oral analgesia, or both (one-way ANOVA, p=0.77).

4.2. Clinical examination of the efficiency of PDT

4.2.1. Efficiency data throughout a four-year treatment period

Eighty seven patients were involved (32 females, 55 males, mean age=72 years, age range=43-92 years). The locations of the tumors were as follows: head and neck region: 111 (cheeks: 22, forehead: 31, temporal area: 12, nose: 18, auricular region: 12, lip: 1, scalp: 11, neck: 4), trunk: 45 (back: 29, chest: 15, abdomen: 1), and extremities: 26 (shoulders: 13, arms: 6, hands: 5, shin: 1, thigh: 1).

The two photosensitizers, ALA and MAL, were equally efficient in the first and second treatments. In the ALA-PDT group, complete remission was attained in 62,7 % in BCC and 57,5% in AK, and incomplete remission in 17,6% in BBC and 23,4% in AK, while there was no response in 19,6% in BCC and 19,2% in AK. For MAL-PDT, the level of complete remission was 67,4% in BCC, and 60,6% in AK, while that of incomplete remission was 19,6 % in BCC, and 27,3 % in AK, and there was no response in 13 % in BCC, and in

12,1% in AK ***(Table 1.)***. A second treatment was required for 71 tumors, but because of non-compliance, treatment could only be performed in 63 cases. Cases where there was no response or there was incomplete remission after the second PDT session were followed by surgical excision (29 tumors) or a third PDT session (10 tumors).

Efficacy analysis					
Photosensitizer	Therapeutic result	BCC	AK	BD	total
ALA	NR	10 (19.6%)	9 (19.15%)	0 (0%)	19 (18.45%)
	IR	9 (17.64%)	11 (23.4%)	4 (80%)	24 (23.3%)
	CR	32 (62.74%)	27 (57.45%)	1 (20%)	60 (58.25%)
		n= 51	n=47	n=5	n=103
MAL	NR	6 (13%)	4 (12.1%)	0 (0%)	10 (12.66%)
	IR	9 (19.56%)	9 (27.3%)	0 (0%)	18 (22.78%)
	CR	31 (67.4%)	20 (60.6%)	0 (0%)	51 (64.56%)
		n=46	n=33	n=0	n=79

Table 1. Efficacy of PDT with two different photosensitizers. NR - no response, IR - incomplete remission, CR - complete remission.

The well-known side-effects were observed in all cases, irrespective of which photosensitizer was used: 1-2 hours after the treatment, erythema, crusting, a serous discharge, oedema and sterile pustule formation. An antiseptic cooling cream was applied locally to moderate these side-effects.

4.2.2. In vivo confocal reflectance microscopy with three different wavelength lasers before and after photosensitization and immediately after photodynamic therapy

For routine RCM testing, the 785-nm laser is used most widely. But since two other laser wavelengths (658 nm, 488 nm) were also available for us we first started examining whether there was a difference between the images captured by the three different wavelength lasers and whether the photosensitizer ALA cream causes any change on the RCM images or not. Of one selected actinic keratosis, images were taken with all three lasers before applying ALA-containing cream, immediately after removing the cream and immediately after illumination. The three different laser images showed no significant difference, and the deepest study of the dermis could be done with the 785 nm laser. Based on this, hereafter we worked with this laser. None of the lasers showed reflectivity associated with the applied ALA.

4.2.3. Reflectance confocal microscopy before photodynamic therapy

In the further phase of the study 12 patients were enrolled, with each patient we chose and treated one selected epithelial tumor according to the study. The PDT-treated lesions were distributed as follows: 4 actinic keratoses, 7 superficial basal cell carcinomas, 1 Bowen's disease. The clinical diagnosis was confirmed by a confocal microscopic examination prior to treatment. In 7 cases a biopsy were also made also prior to this study. All of the lesions showed typical signs appropriate for the three above mentioned diagnoses with RCM ***(Fig.9., Fig.10.)*** and histopathological examination correlated well with the RCM finding.

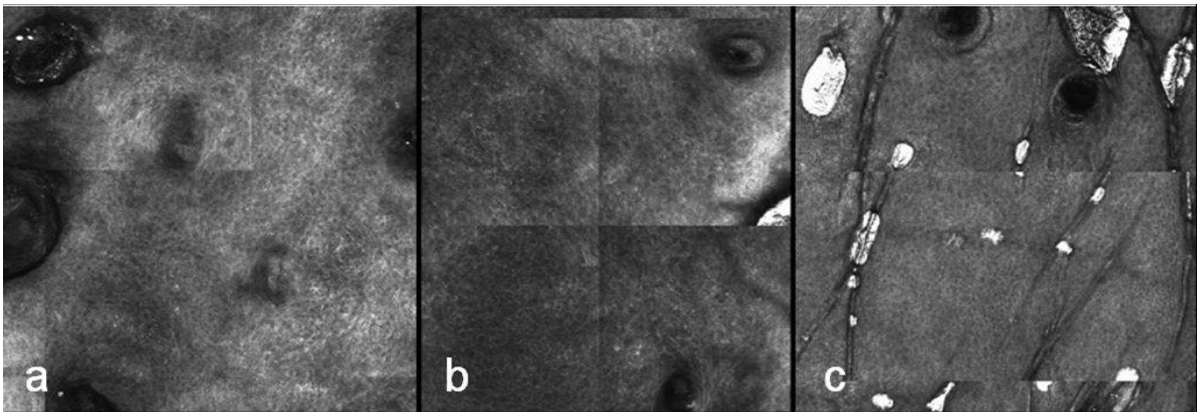

Figure 9. In vivo reflectance confocal microscopy of an actinic keratosis
a.) RCM picture of AK before treatment: irregular honeycomb pattern (architectural disarray of the epidermis, cellular nuclear pleomorphism)
b.) RCM picture of AK immediately after illumination: spongiosis, exocytosis (presence of inflammatory cells)
c.) RCM picture of AK four weeks after PDT: complete remission, regular honeycomb pattern

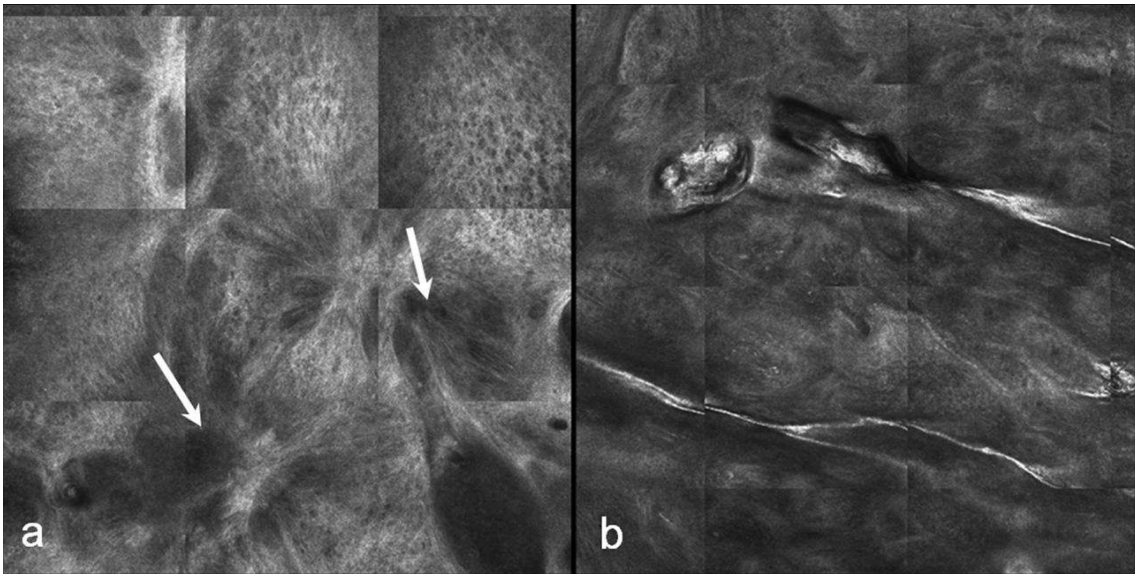

Figure 10. In vivo reflectance confocal microscopy of a superficial basal cell carcinoma
a.) RCM picture of superficial BCC before treatment (lobulated tumor cell nests, elongated bunches)
b.) Superficial BCC four weeks after PDT (regular honeycombed pattern)

4.2.4. Fluorescence diagnosis after incubation with the photosensitizer before illumination

After a 3-hour incubation with the photosensitizer, in all cases the fluorescence in UV light was shown clearly in the area of the tumor due to the accumulation of PPIX ***(Fig.11.)***.

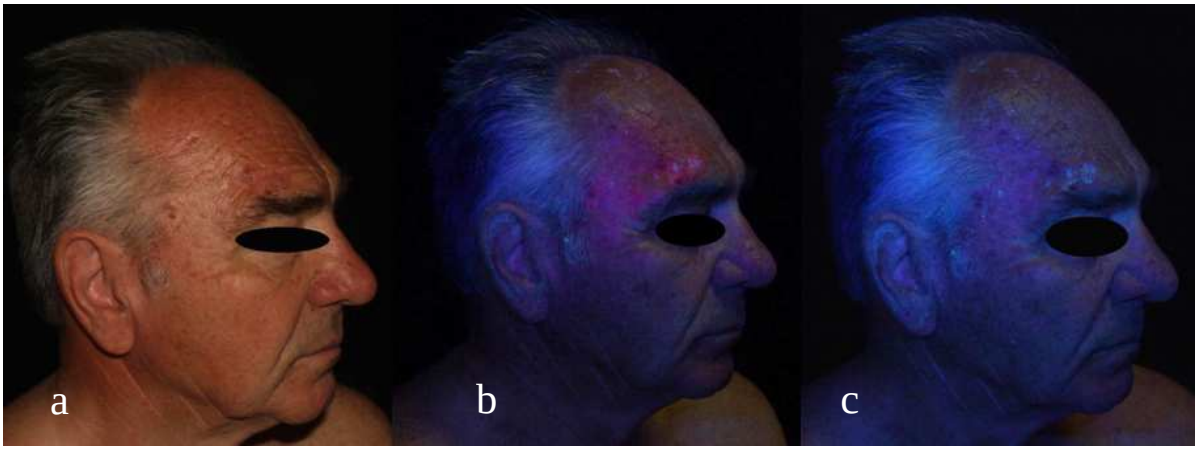

Figure 11. Photodynamic diagnosis of an actinic keratosis

a.) Actinic keratosis above the eyebrow

b.) After 3 hours of ALA incubation, detectable coral red fluorescence of the accumulated PPIX in UV light

c.) After illumination with 630 nm wavelength red light the PPIX was degraded thus the fluorescence disappeared

4.2.5. Examination of the immediate effects of PDT with RCM after illumination

Immediately after illumination on the dermoscopic picture explicit erythema was seen, with RCM edema and vasodilatation in the area of the stratum granulosum and spinosum showed, contours of the epithelial cells became more explicit, and a few inflammatory cells appeared ***(Fig. 9.b.)***.

4.2.6. RCM at the follow up visit, four weeks after photodynamic therapy

The efficacy of one time PDT was evaluated 4 weeks after treatment, 12 patients were included into the study. Unfortunately one patient with actinic keratosis did not recur for follow-up. In the remaining 11 patients we observed complete remission (CR) in 8 cases (72.7%), incomplete remission in two cases (18.2%) and treatment failure in one patient. Breaking down for diagnosis the efficiency results were as follows: 100% (3/3) complete remission for three AK, one (1/1) partial remission for BD, and 71.4% (5/7) complete remission for sBCCs. One (1/7) sBCC showed incomplete remission and one (1/7) did not respond to treatment at all ***(Fig. 9., Fig. 10.)***. Dermoscopic images of an actinic keratosis and a superficial basal cell carcinoma before and after PDT are shown on ***Fig.12.*** and ***Fig.13***.

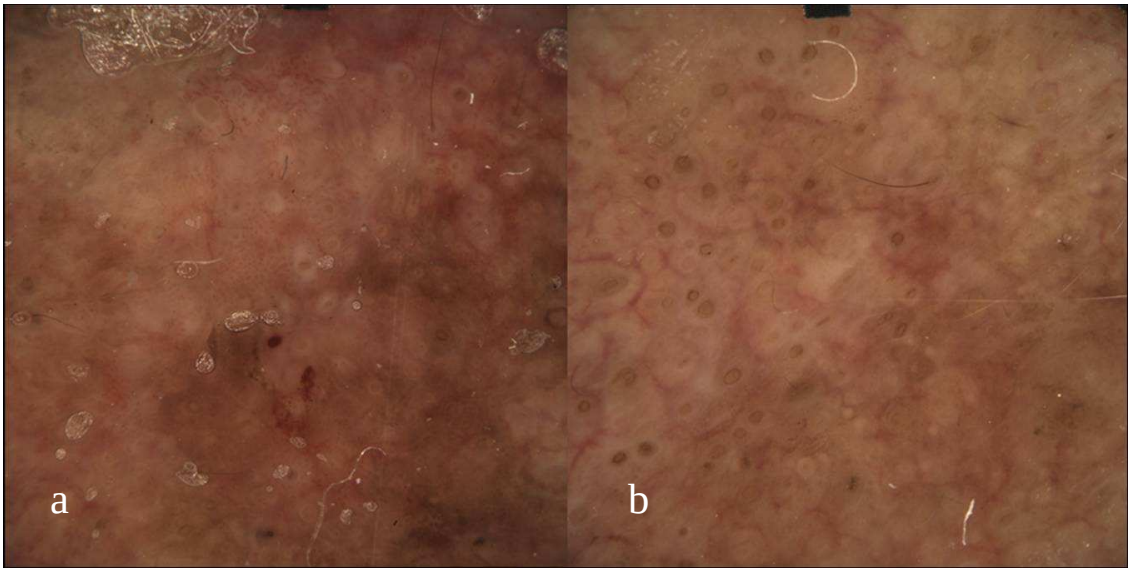

Figure 12. Dermoscopic picture of an actinic keratosis before (a) and four weeks after (b) PDT

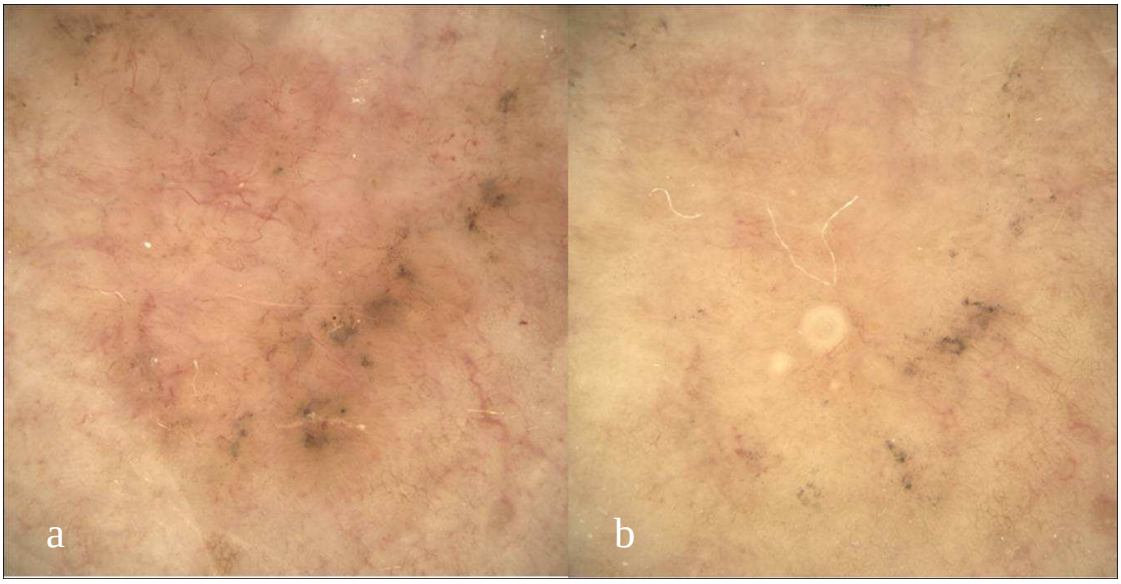

Figure 13. Dermoscopic picture of a superficial basal cell carcinoma before (a) and four weeks after (b) PDT

4.2.7. Fluorescence diagnosis for the assessment of treatment efficiency

At 4-weeks follow-up a photos were taken and confocal microscope imaging was performed. In one case of actinic keratosis, ALA was applied once again on the previously treated area for 3 hours. After photosensitization, we examined the extent of fluorescence under UV light. In this case of complete remission, a minimal, diffuse fluorescence was detected, which was even less explicit on the treated area than on the surrounding skin ***(Fig.14.)***.

In one case of Bowen's disease and one case of superficial BCC we found at follow-up clinically and with RCM incomplete remission and decided to perform PDT a second time. In these two cases a well-defined fluorescence was detectable in the lesions after ALA incubation (not shown).

In addition, one case of basal cell carcinoma proved to be more infiltrating at follow-up, which was supported by RCM. Therefore we decided to perform complete surgical excision in this case.

Figure 14. Photodynamic diagnosis of an actinic keratosis at the follow up visit 4 weeks after PDT

a.) Clinically complete remission

b.) After 3 hours of ALA incubation only minimal fluorescence is detectable

5. DISCUSSION

5.1. Advantages of PDT

When treating epithelial skin tumors, in the choice of therapeutic treatment the primary consideration is efficiency. However, we should bear in mind the cosmetic outcome and the expectations of the patient towards treatment as well. The greatest advantages of PDT are that it is non-invasive, it can be repeated unrestrictedly and it results in an excellent cosmetic outcome. Its proven efficiency rivals surgical treatment, and its cosmetic result is much more favorable. The especially good cosmetic result is due to the fact that the fibroblasts do not accumulate the photosensitizer substance, thus eliminating treatment associated scarring [54]. The occurrence of pigmentation disorders due to PDT is also not typical. After treatment, skin texture changes and skin bumps disappear, wrinkles become more superficial and previously existing pigmentation disappears, and finally the skin rejuvenates. Nowadays this is consciously used with rejuvenating treatments [55]. In contrast to surgical treatment, PDT allows treatment of those precancerous cells, not yet visible to the naked eye, but with an already changed DNA. In case of an incomplete regression the treatment can be repeated an unlimited number of times, and PDT (as opposed to radiation therapy) does not effect the possibility of subsequent surgery.

5.2. PDT as an evidence based treatment method

By now an international consensus has been born about the treatment with PDT, detailed treatment protocols have been made with the effect that photodynamic therapy is a modern, practical, evidence-based approach for the prevention and treatment of epithelial tumors (actinic keratosis, superficial and nodular basal cell carcinoma, Bowen disease) [31;56]. Prevention is primarily important with organ transplant patients who are at particular risk of developing invasive skin tumors [57]. PDT has proved to be effective in AK, with excellent cosmetic results, the evidence level of this is AI (there is a good evidence to support the use of the procedure obtained of at least one, properly designed, randomized controlled trial). It is also of an AI-evidence-level that for Bowen disease PDT is at least as effective as cryotherapy or 5-fluorouracil, PDT, however it has less side-effects. In the case of squamous cell carcinomas there is a lack of evidence for the routine application of PDT: the evidence-level

is CIIiii (evidence obtained from multiple time series with or without intervention). In the case of sBCC, PDT has proven to be effective, in addition to an excellent cosmetic result with the other advantage of the simplicity of the treatment of multiplex or extensive lesions (AI-evidence level). In the case of nodular basal cell carcinoma the treatment of tumors only thinner than 2 mm is justified by PDT (AI-evidence level) [31].

5.3. Pain during PDT, the main limiting factor

Side-effects of PDT are usually moderate and mostly self-limiting. Intense pain during and after treatment sessions is the most important undesired reaction, often causing premature termination of treatment, and thus, limited efficiency. Methods to improve or to alleviate PDT related discomfort are, therefore, of paramount significance for the future of photodynamic therapy.

5.3.1. Factors affecting PDT related pain, pain relief

PDT associated pain is influenced by several intrinsic (patient-related) and extrinsic (treatment-related) factors. The anatomical region, the diagnosis and the size of the lesion, as well as the degree of photoageing in and around the treatment area [58] are significant intrinsic determinants of treatment related pain, whereas the patient's skin type seems to be unimportant [59]. Lesions localized on the scalp or forehead are more sensitive, compared with the trunk and the extremities [59;60]. Patients with AK seem to experience more intense pain than those with BCC (possibly due to more advanced photoageing) and, in general, pain increases with lesion size [59;60]. Age and gender are two intrinsic factors potentially influencing PDT related pain which have been poorly investigated. In some trials gender and age did not influence pain [60-62], but controversial data was also found [63]. In our study increasing age was significantly associated with more pain sensation.

Extrinsic PDT factors, such as the use of analgesics, type of photosensitizer, light source, wavelength and dose, – at least theoretically – provide the possibility to influence treatment related pain. Several studies have reported attempts to moderate PDT-induced pain, but comparative investigations have not yet been performed. Local anaesthetics (Emla cream containing lidocaine and prilocaine [64], 0,3% morphine gel [65], and tetracaine gel [66]) have all proven to be ineffective. Cooling with a wet gauze, thermal water spraying, and 1 week pre-treatment with capsaicin (0,075%) have also been tried, but with either no or limited effect [59].

Pain of the most sensitive anatomical regions, such as the penis or vulva, was successfully controlled by conduction anaesthesia [67]. Cold air analgesia and subcutaneous local infiltrative anaesthesia, also proved valuable [68;69]. Nerve blocks provided effective pain relief during topical PDT for AK on the scalp, forehead or face [70;71], and were superior to cold air analgesia [72].

Pain can also be modulated through the choice of different light sources and wavelengths. Variable pulsed light was found to be less painful than LED light for topical PDT of AKs in a prospective randomized controlled trial [73]. Fritsch et al. reported that PDT with green light was less painful than the use of red light [74]. However, Morton et al. came to the opposite conclusion: despite the lower permeability of green light in the skin, the severity of pain was found to be similar during red light and green light PDT sessions [75]. Contrary to expectations, PDT associated pain is reportedly independent of the administered light dose [59]. Pain related to the treatment is associated with the level of porphyrin accumulation detected before illumination [76].

MAL and ALA are the most widely used topical photosensitizers in the treatment of non-melanoma skin cancer. However, only relatively limited data is available concerning treatment-associated pain using the different photosensitizers. Kasche et al. found that MAL-PDT caused significantly less pain than did ALA-PDT in patients with multiple AKs on the scalp [52]. The reason is probably the greater tumor selectivity of MAL or the fact that ALA, but not MAL, is actively transported to the peripheral nerve endings, triggering nerve stimulation during its excitation [52;77]. Steinbauer et al. also considered the use of ALA, in contrast with MAL, as a factor predictive of higher PDT associated pain [78].

In the present study, we evaluated treatment-associated pain during PDT of non-melanoma skin cancer (AK, BCC and BD) in different anatomical regions, using the two photosensitizers, MAL and ALA. We found that in the sensitive head region MAL-PDT was more tolerable and caused significantly less pain than ALA-PDT. There was a tendency for ALA-PDT to be more painful in all anatomical regions, but in the case of the trunk and the extremities the differences were not significant. PDT of actinic keratosis was significantly more painful than treatment of basal cell carcinomas. Our findings are in accordance with previous studies in regards to the connection between diagnosis and therapy-induced pain. Moreover, the observed significant difference between MAL- and ALA-PDT associated pain in the BCC and AK groups, is in line with the previous observation that MAL-PDT causes less pain. In the case of nine out of ten patients, whose treatment was prematurely terminated due to intolerable pain, the photosensitizer used was ALA. Certainly, one could hypothesize

that the observed differences in pain levels could at least in part be attributed to the different ALA and MAL concentrations, however, the similar effectiveness of both photosensitizers does not support this theory. No difference was detected between the methods of pain relief used: cooling the skin with wet gauze during treatment, oral analgesia (paracetamol) before treatment, or both.

5.3.2. Lack of adequate pain relief

Adequate pain relief during PDT presents a difficulty. Pain related to the treatment is associated with the level of porphyrin accumulation detected before illumination [76]. Among the methods of pain relief, many did not prove to be sufficiently effective. Recently, the most common effective procedure was the acceleration of cold air during illumination. A retrospective comparative study with 100 patients showed that the cold air acceleration during illumination reduced PPIX degradation, but also the effectiveness of the treatment [79]. This unexpected result may decrease the degree in which this otherwise very effective pain relief method is used.

5.3.3. Our experiences of PDT-associated pain

Our data confirms that while ALA- and MAL-PDT are both equally highly effective for the treatment of non-melanoma skin cancer, MAL-PDT is better tolerated. The lower level of treatment associated pain suggests better suitability of MAL-PDT for the treatment of sensitive anatomical regions or for patients at risk of more pain (e.g. larger lesions or diagnosis of AK, photoageing or field cancerization). Large, well-designed, and well-controlled studies are clearly required to confirm the present observations and to promote the development of an optimally efficient strategy with which to reduce PDT-related pain.

5.4. Adjuvant methods of monitoring the efficiency of PDT

5.4.1. Protoporhyrin accumulation and protoporphyrin photobleaching

Monitoring the level of PPIX accumulation became possible with the help of fluorescence imaging systems before applying the photosensitizer, immediately after incubation and also after illumination. It had been believed that the absolute level of PPIX accumulating in cells is the crucial factor for the efficacy of PDT. Today it seems that it is not the amount of PPIX which is the decisive factor but its decrease during illumination [80]. The more PPIX decays due to illumination (photobleaching), the more oxygen free-radical is formed which is directly proportional to cell destruction and to treatment efficiency. The increase of fluorescence during incubation time, and its decrease during illumination is only explicit in neoplastic areas, as fluorescence of normal tissue does not change during treatment [81]. The concentration of PPIX at first and second PDT – after photosensitization but before illumination – has been studied by some authors. No consistent results have been reached. Tyrell et al. found significantly less PPIX accumulation and dissipation in 25 examined lesions during second PDT than during first treatment [81]. Fluorescence, thus changes in the level of PPIX, did not depend on gender, age and type of epithelial tumor and even not on the size of tumor [81]. Sandberg et al. studied 35 basal cell carcinomas 1 week after first treatment. They found during second treatment an even greater contrast in fluorescence intensity [40]. The explanation for this could be the fact that the damaged skin barrier during first treatment and the inflammatory processes after treatment allow for a greater accumulation of porphyrin [40]. Pain relief during illumination has always been a problem. In recent years the most widely used procedure has been cold air acceleration. A retrospective comparative study with 100 patients showed that cold air acceleration during illumination reduced PPIX degradation and thus the efficiency of treatment [79]. Clinical response to PDT is also reduced in lesions with acral localization [82]. So Tyrell et al. found lower PPIX accumulation and destruction of PPIX fluorescence in acral actinic keratoses [82].

5.4.2. Conventional methods and subsequent non-invasive diagnostic procedures for the assessment of therapeutic results of PDT

Methods for photodynamic diagnosis and imaging have been developed only in the past two decades. Photodynamic diagnosis – a method previously used only for verifying the effectiveness of photodynamic therapy – has recently reached a more independent, more important role, as PDD became more quantifiable [39]**.** It has been proved that fluorescence imaging produced highly sensitive and specific results compared with histological examination. Unfortunately randomized controlled clinical trials are still lacking, but preliminary studies seem to be quite promising [36]. To project this to the level of practical application it seems that fluorescence diagnosis will be very much suited for early, cost-effective and reliable monitoring and simplifying Mohs surgery. This modified microsurgical method is the accepted and most effective method for the removal of high-risk basal cell carcinomas on the face and ears. The procedure of Mohs surgery, however, does involve very high costs, is time consuming and requires the presence of specially trained personnel. Imaging procedures suitable for speeding up the implementation of MMS - through early and more precise determination the edges of the tumor - had previously been sought after. But as mentioned above, these methods are difficult to achieve[36]. Before surgery a Mohs surgeon - in possession of a fluorescence image - is able to plan lines of excision not merely on empirical basis (eye and palpation) but with almost absolute certainty. While photodynamic therapy is also applied for the treatment of eye-, mouth-, esophagus- and bladder-tumors, photodynamic diagnosis is used, besides skin neoplasias,mostly for bladder neoplasias. The borders of invasive bladder carcinoma and even of carcinoma in situ lesions are made visible by fluorescence diagnosis, moreover FD proved to be more precise than the traditional endoscopic examination [83]. Fluorescence detection of peritoneal metastases of gastric cancer has also been reported. [84].

In the field of photodynamic diagnosis and therapy one of the biggest challenges is to facilitate the penetration of the photosensitizer molecules, and to increase the penetration of the exciting light. The development of several second-generation photosensitizer molecules, whose absorption peaks fall within the red-infrared spectrum, is in progress. They will be able to be excited by larger wavelength light sources, which penetrate even deeper into the skin. The efficiency of currently used photosensitizers is limited due to their hydrophobic nature. To achieve better penetration and higher fluorescence ratio photosensitizers will have to be

combined with other molecules. Chlorin e6 together with hydrophilic polyvinylpyrrolidone (PVP) resulted in a higher fluorescence contrast as alone or even in combination with DMSO [85]. Animal tests and early human studies with angiosarcomas are promising [85]. Novel nanostructural and liposomal photosensitizers have also been developed which showed in vitro higher photodynamic effect on human colorectal carcinoma and prostate cancer cell lines [86]. In skin carcinoma cells pretreatment with low-dose methotrexate before ALA incubation increases the intracellular PPIX level 2 to 4-fold in comparison with normal keratinocytes [87]. Vitamine D3 also enhances the apoptotic response of epithelial tumors to ALA PDT in vitro by altering the expression of porphyrin-synthesis enzymes [88]. By the increase of light dose through the extension of illumination time the depth of penetration and treatment efficiency will be enhanced [82].

In the literature the 785-nm laser is used most commonly for routine RCM examination. Our confocal microscope has three different lasers (488 nm, 658 nm, 785 nm) which we decided to compare. Images were taken with all three available lasers of one selected actinic keratosis before applying ALA-cream, immediately after removing the cream and finally immediately after illumination. The three different laser images showed no significant difference. The deepest examination of the dermis was made using the 785 nm laser. Based on this experience we continued our worked with the 785nm laser. This way our study is also more comparable with previously published data. We did not see any reflectivity associated with ALA-cream using any of the three lasers. We believe that the 658 nm (red) and 488 nm (blue) lasers do not excite PPIX in a sufficient amount. So confocal microscopy with these three lasers seems to be not suitable for the detection of PPIX accumulation in the skin. After application of ALA-cream there was neither a detectable erythema nor had the patients any complaints when the pictures were taken. Obviously the wavelength range of the blue and red light necessary for the activation of PPIX seems to be too narrow (405 and 630 nm). These wavelengths also seem to differ from the laser wavelength of RCM. In addition, the exposition time was rather short and the examined area was also relatively small (8x8 mm).

Only those cases were regarded as complete remission when the above mentioned deviations and characteristics of epithelial tumors had disappeared ***(Fig. 12.C., Fig. 14.B.)*** and only a residual solar elastosis was observed. Our efficacy data of one-time PDT, measured clinically, and using confocal microscopy, were identical to previously reported data. According to publications for actinic keratosis when PDT was performed once, complete remission was obtained 69-100%, in the case of superficial basal cell carcinoma 86-100% [31].

At follow-up the most accepted method to judge the effectiveness of treatment and possible recurrence was physical examination (visual, both dermoscopic and tactile). Photodynamic fluorescence, enabling to determine the actual size of the tumor, facilitates to get a more accurate assessment of therapeutic response and possible recurrence. If complete remission cannot be precisely judged at follow-up, a 3-hour MAL or ALA photosensitizer incubation and a fluorescence test under UV light may be performed. If a well-defined area with stronger fluorescence than the surrounding area is detected PDT may be repeated. In comparison with ALA using MAL photosensitization a higher contrast is achieved between tumor and surrounding healthy tissue [40]. Therefore MAL is preferable for fluorescent diagnostics. MAL's higher tumor selectivity has been known for quite some time [43;44]. However several published data as well as our own studies suggest that ALA-PDD makes the edges of the tumors perfectly visible, and the accuracy of ALA-PDD also correlates very well with histological examination [89].

Only one publication reported using reflectance confocal microscopic examination for the assessment of therapeutic response subsequent to photodynamic therapy of skin tumors [90]. They examined the response of basal cell carcinomas to PDT [90] in patients who suffered from Gorlin's syndrome or from Xeroderma pigmentosum. In genodermatoses with predisposition to skin cancer, non-invasive diagnosis and treatment methods may greatly improve patient's quality of life. Without PDD and PDT patients with such genodermatoses would undergo a number of biopsies and surgical procedures [90].

To conclude, in the present study the applicability of two non-invasive diagnostic methods was examined before and after photodynamic therapy. In vivo confocal microscopy, supplemented with a dermoscopic examination can give an even more precise diagnosis and is useful for patient follow-up [91]. For the extremely precise determination of the edges of a tumor, fluorescence diagnostics is helpful.

6. SUMMARY

In the treatment of non-melanoma skin cancer beside surgical excision several alternatives exist. Photodynamic therapy (PDT) is a non-invasive, indefinitely repeatable, efficient, and evidence based treatment of choice in certain types of skin tumors, with an excellent cosmetic outcome. By PDT protoporphyrin IX (PPIX) is generated from the exogenous photosensitizer – 5-aminolevulinic acid (ALA) and methyl-aminolevulinate (MAL) – applied to the skin surface. PPIX is excited by light with the appropriate wavelength

after which it returns to its base state while reactive oxygen species (ROS) are formed leading to cell death in rapidly proliferating cells.

Therapy-related pain is the most frequent side-effect, which often limits the duration and thus efficiency of treatment. Patients usually report a cumulative burning sensation during illumination that becomes intense within a few minutes after the starting of the procedure. In some cases, the pain may become so severe that the illumination must be stopped prematurely, with the result that the applied light dose, and the PPIX formation are insufficient, and the therapeutic effect is inadequate. Pain and pain relief during PDT is therefore a very important factor. Adequate and simple pain relief is difficult.

MAL and ALA are the most widely used topical photosensitizers in the treatment of non-melanoma skin cancer. However, only relatively few data are available concerning treatment-associated pain using the different photosensitizers.

In the present study, we evaluated treatment-associated pain during PDT of non-melanoma skin cancer (AK, BCC and BD) in different anatomical regions, using the two photosensitizers, MAL and ALA. We found that in the sensitive head region MAL-PDT was more tolerable and caused significantly less pain than ALA-PDT. There was a tendency for ALA-PDT to be more painful in all anatomical regions, but on the trunk and the extremities the differences were not significant. PDT of actinic keratoses was significantly more painful than treatment of basal cell carcinomas. Our findings are in accordance with previous studies about the relation between the diagnosis and the therapy-induced pain.

Our data confirm that while ALA- and MAL-PDT are both highly effective for the treatment of non-melanoma skin cancer, MAL-PDT is better tolerated. The lower level of treatment associated pain suggests better applicability of MAL-PDT in sensitive anatomical regions or for patients at risk of more pain (e.g. larger lesions or diagnosis of AK, photoageing or field cancerization).

Excited PPIX - as well as neoplastic cells containing it large amounts – shows fluorescence with ultraviolet light, which may be used for diagnostics. This process is suitable for the exact determination of tumor margins or judgement of the therapeutic result and may also be combined with other invasive or non-invasive therapeutic methods. In vivo reflectance confocal microscopy (RCM) is a non-invasive, non-stressful, repeatable method, suitable for patient follow up, skin cancer diagnostic and in certain cases for tumor extension measurement. Patients with skin lesions suitable for PDT - actinic keratosis, superficial basal cell carcinoma or Bowen disease – were enrolled in the other part of the study. We performed fluorescent diagnosis (FD) between steps of PDT and RCM before and after PDT. 4 weeks

after the treatment we evaluated the therapeutic result with the help of confocal microscopy and in certain cases fluorescence diagnosis too. In vivo confocal reflectance microscopy is an appropriate non-invasive process to confirm the diagnosis of skin tumours and to investigate the therapeutic result.

Photodynamic therapy is an effective method in treating certain epithelial tumors, it is non-invasive and can be repeated indefinitely with excellent cosmetic results. Each year its dermatological use becomes more and more widespread. Supplemented with fluorescence diagnostics PDT is highly suitable for an effective, even independent treatment of these tumors or for a treatment combined with surgical methods and for a more precise assessment of the effectiveness of the treatment.

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