Comparison of PpIX accumulation and destruction during methyl-aminolevulinate photodynamic therapy (MAL-PDT) of skin tumours located at acral and non-acral sites

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What's already known about this topic?

Methyl-aminolevulinate photodynamic therapy (MAL-PDT) is known to be successful in the treatment of non-melanoma skin cancers (NMSC) and pre-cancerous lesions. However studies have demonstrated that efficacy of this treatment is reduced when these skin lesions are located on the body's extremities at acral sites.

What does this study add?

This study provides further evidence of the disparity between the response of dermatological skin lesions located at acral and non-acral sites to MAL-PDT treatment. For the first time this study monitors the changes in PpIX fluorescence during MAL-PDT treatment of (pre)cancerous skin lesions located within acral and non-acral sites. Comparison of changes in PpIX fluorescence within lesions at these sites furthers our understanding of the poorer response witnessed at acral sites.

Abstract

Background Topical photodynamic therapy is successful in the treatment of non-melanoma skin cancers and associated pre-cancers, but efficacy is significantly reduced in actinic keratosis lesions not located on the face or scalp.

Objectives The purpose of this investigation was to compare the changes in protoporphyrin IX fluorescence in lesions undergoing routine methyl-aminolevulinate (MAL) PDT and the clinical outcome observed three months after treatment in lesions located at acral and non-acral sites.

Patients/methods This study was a non-interventional, non-randomised, observational study, which monitored changes in PpIX fluorescence in 200 lesions during standard dermatological MAL-PDT. These data were subsequently analysed in terms of lesions located at acral and non-acral sites.

Results Clinical clearance was significantly reduced ($P<0.01$) in acral skin lesions when compared to lesions located at non-acral sites. The accumulation and destruction of PpIX fluorescence was significantly reduced in these acrally located lesions ($P<0.05$ and $P<0.001$ respectively).

Conclusions These data suggest that reduced PpIX accumulation and the subsequent reduction in PpIX photobleaching within acral lesions results in the reduced responsiveness of these lesions to MAL-PDT. Future work should therefore aim to improve photosensitiser accumulation/photobleaching within lesions located at acral sites.

Introduction

Photodynamic therapy (PDT) is capable of the selective ablation of malignant tissue via the localised production of singlet oxygen and other reactive oxygen species (ROS) and therefore this treatment is currently utilised in the treatment of a wide range of carcinomas[1]. PDT requires the presence of three critical components, a photosensitiser, light of the appropriate wavelength and oxygen within the tissue[2]. A particular niche for PDT has been found in the treatment of certain non-melanoma skin cancers (NMSC) (e.g. basal cell carcinoma (BCC)) and other precancerous skin conditions (e.g. actinic keratosis (AK) and Bowen's disease (BD)). This topical treatment involves the application of a prodrug, usually either aminolaevulinic acid (ALA; licensed in the USA) or methyl-aminolevulinate (MAL; licensed in the UK), which are converted via the haem biosynthesis pathway to the endogenous photosensitiser, protoporphyrin IX (PpIX). Tumour cells preferentially accumulate PpIX due to an increased uptake of MAL as a result of their disrupted stratum corneum, up-regulation of the haem biosynthesis pathway and differences within porphobilinogen deaminase and ferrochelatase expression[1,3].

Currently available clinical data indicates high complete response rates are observed when treating these dermatological lesions with topical PDT and these response rates are comparable to the standard treatment modalities of cryosurgery and surgical excision [4-9]. Additionally ALA/MAL-PDT enables the successful treatment of large or multiple lesions which present a greater challenge for more conventional treatments and furthermore topical PDT treatment of these lesions provides a significantly improved cosmetic outcome [4-9].

In the UK, MAL-PDT is currently licensed for the treatment of i) thin or non-hyperkeratotic and non-pigmented AK on the face/scalp when other therapies are considered less appropriate; ii) superficial/nodular BCC unsuitable for other therapies due to possible treatment morbidity and poor cosmesis; iii) Bowen's disease when surgery is not considered appropriate[10-12]. To date limited studies have looked at the treatment of acral AK lesions (acral – of, relating to, or affecting peripheral parts, such as limbs, fingers or ears) with MAL-PDT. A multi-centre randomised control trial indicated that MAL-PDT treatment of AK lesions located in the extremities was less effective than cryosurgery, although this trial demonstrated a significantly improved cosmetic outcome and patient preference for MAL-PDT treatment[6]. The improved response of facial AK lesions was also noted by a number of investigators utilising both ALA-PDT[13-17] and MAL-PDT[18]. No studies to date have compared the response of BCC and BD lesions located at acral and non-acral sites to determine if the variation in clinical response that exists in AK also occurs within these lesions.

The exact reason for the limited efficacy of PpIX mediated PDT in the treatment of acral AK is unknown, although greater lesion thickness, reduced skin temperature and poorer pro-drug absorption as a result of fewer pilocebaceous units within the acral regions have been postulated to play a role[6]. This study endeavours to elucidate the basis of this poorer response by utilising the fluorescent properties of the photosensitiser PpIX to follow the accumulation of the photosensitiser following MAL application and PpIX photobleaching during light irradiation. The changes in PpIX and the clinical response of AK, BD and superficial BCC (sBCC) at acral and non-acral locations has therefore been investigated.

Materials and Methods

Fluorescence imaging

PpIX fluorescence was determined using a previously validated[19], non-invasive imaging system (Dyaderm, Biocam, Germany). Tyrrell *et al*., 2010 previously described and discussed this system extensively in terms of the imaging apparatus and how to utilise the system appropriately to acquire reproducible images[19]. However in brief, the system consists of a Xenon light source with a custom bandpass filter (370–440 nm) and a 12-bit Sony charge coupled device (CCD) camera combined in one adjustable arm coupled to a computer equipped with image capturing software (Dyaderm Pro v2, Biocam, Germany). The flash light emits seven light pulses per second to the skin and the light that returns to the skin is collected by the CCD camera (exposure time 100 µs) equipped with a Schott GG 455 longpass filter which filters out the excitation light. Excited PpIX emits light in the form of fluorescence in the red spectrum and the red pixels of the CCD camera are used to generate a fluorescence image alongside a simultaneously collected colour image, both of which are processed by the system in real time. Images from a synthetic PpIX (630 nm) fluorescent standard (Biocam, Germany) and the patients were recorded in bitmap format and exported into NIH ImageJ software (http://rsb.info.nih.gov/ij/) enabling raw data to be analysed. It should also be noted that the fluorescence images acquired by the imaging system utilised have previously been demonstrated to correlate with the PpIX content of the tissue following chemical extraction [20].

Clinical data collection

All patients attending the PDT clinic in the Dermatology Department, Royal Cornwall Hospital for routine PDT treatment were provided with verbal and written information about the study prior to giving written consent before participating in this ethically approved (Cornwall and Plymouth Research Ethics Committee) fluorescence imaging study. This investigation was a non-interventional, non-randomised, observational study, which monitored all consenting patients over a two year period (January 2008 – December 2009) with licensed indications (AK, BD and sBCC) for MAL-PDT in the UK treated at this clinic[11]. The majority of BCC and BD lesions were biopsy proven (85%) prior to referral for MAL-PDT treatment, whilst AK lesions were generally referred at the Consultant Dermatologist's discretion. This study monitored lesions located at a variety of different anatomical sites including acral and non-acral regions of skin.

Two hundred patients (112 males and 88 females; age range 42-98, average 73 years) were recruited and one lesion was monitored in each patient to limit statistical error. There was an approximately equal split in terms of the lesion localisation (104 non-acral and 96 acral, with acral defined as of, relating to, or affecting peripheral parts, such as limbs, fingers or ears) and these were further subdivided into the histologically distinct lesions (83 AK (with individual lesions monitored) (52 non-acral and 31 acral), 75 BD (21 non-acral and 54 acral) and 42 sBCC (31 non-acral and 11 acral). The lesions were treated as per the standard clinical protocol, with overlying crust being removed from the lesion prior to the application of a thin layer (1 mm thick with a border of 5 mm around the visible lesion) of the topical MAL cream (160 mg/g MAL, commercially known as Metvix®, Galderma, UK). The lesion was then occluded from the light for a period of three hours. After the allotted time the lesion

was irradiated with a red light (Aktilite, Galderma, UK; 635 nm +/- 5 nm; 37 Jcm^{-2}; 90 $mWcm^{-2}$) which was positioned between 5 and 8 centimetres away from the lesion. It should be noted that the lesions received a second identical treatment nine days later. This is different to the standard normal seven day re-treatment advised for MAL-PDT. This slightly longer time period is employed by our department as it better suits our clinical setup, where new patients are seen on Tuesdays for their first PDT session and patients return on the subsequent Thursday for a second PDT treatment (if required).

The lesions were imaged at multiple time points during standard PDT treatment, specifically prior to the application of MAL, after the three hour MAL application period and immediately following light irradiation. All images were taken in accordance with our previously derived standardised operating procedure which enabled reproducible images to be acquired by limiting the other factors potentially altering image acquisition[19]. The fluorescence intensity within images (arbitrary units (AU)) were then analysed at a consistent pixel position using ImageJ software to follow the changes in PpIX fluorescence within the lesion at the different points in the treatment.

Outcome at three months

All patients attended an outpatient clinic three months after their last PDT treatment and the lesions were visually assessed by a Consultant Dermatologist who was blinded to the fluorescence imaging results. The outcome reported was based on the initial assessment of the lesion entered in the notes, which included details of the lesion size and an image of the lesion prior to treatment. If no clinical evidence of the tumour remained at three months then the lesions were considered to have

undergone complete clinical clearance. Lesions that were observed to have decreased in size but where (pre)cancerous cells clearly remained were reported to have undergone a partial clearance. Lesions which remained unaltered following the one or two PDT treatments they had received were reported as no clearance.

Data analysis

The clinical outcomes recorded were compared for acral and non acral skin for the entire data set and for each histologically distinct lesion via the z-test for two proportions. For each patient the percentage change in fluorescence following the three hour MAL application (which reflects PpIX accumulation) and after light irradiation (which reflects PpIX photobleaching) were calculated. These values were compared for acral and non-acral skin via the unpaired t-test. Direct comparison was also made between fluorescence accumulation and dissipation via the paired t-test. The histologically distinct lesions were compared by the two-way ANOVA.

Results

Lesions treated with MAL-PDT located at non-acral sites (n=104) were observed to undergo statistically greater changes in fluorescence after the three hour MAL application ($P<0.05$; Fig. 1a) and following the light irradiation of the lesion ($P<0.001$; Fig. 1b) than lesions located at acral sites (n=96).

When the histologically distinct lesions were considered individually the change in fluorescence following the application of MAL was noted to be statistically reduced in acral AK lesions ($P<0.01$; Fig. 1a) when compared to non-acral AK lesions. In contrast the accumulation of fluorescence following MAL application in BD and sBCC were similar for acral and non-acral sites ($P=0.633$ and $P=0.699$; Fig. 1a). Acral AK lesions were also observed to undergo a significantly reduced percentage change in fluorescence during light irradiation ($P<0.05$) than observed with the non-acral AK lesions. Although the acral BD appeared to undergo reduced photobleaching during light irradiation this difference did not reach significance over non-acral lesions ($P=0.081$) and no significant difference was observed between acral and non acral sBCC ($P=0.543$; Fig. 1b).

Two-way ANOVA analysis indicated no significant difference between lesion type in terms of the percentage change in fluorescence either after MAL application or during light irradiation ($P=0.938$ and $P=0.141$ respectively). Significance only arose from comparison of the different anatomical locations treated.

Previous studies within this group have indicated that the level of PpIX accumulated after MAL application normally approximates to the level of PpIX photobleached during light irradiation [21]. Paired t-tests of the combined lesion types demonstrated that both non-acral and acrally located lesions underwent a significantly greater

change in fluorescence after MAL application than during light irradiation ($P<0.05$ and $P<0.001$ respectively; Fig. 2). Interestingly, when considered separately only acral BD lesions demonstrated significantly greater ($P<0.001$; Fig. 2) PpIX accumulation than PpIX photobleaching. The percentage change in fluorescence after MAL application and during light irradiation was similar in non-acral and acral AK lesions ($P=0.237$ and $P=0.516$ respectively), non acral BD lesions ($P=0.205$) and non-acral and acral sBCC lesions ($P=0.217$ and $P=0.120$) (Fig. 2).

A significantly lower percentage of acral lesions were observed to undergo complete clinical clearance when compared with non-acral lesions (68% versus 85%; $P<0.010$; Fig. 3). This was also true for AK lesions alone, with 85% of non-acral AK lesions been completely clear after three months and only 61% of acral AK undergoing complete clearance ($P<0.05$; Fig. 3). No significant difference was observed between the clearance rates in non-acral and acral BD (81% versus 69%) and sBCC lesions (87% versus 82%) (Fig. 3).

Discussion

MAL-PDT has been demonstrated to be successful in the treatment of NMSC and associated pre-cancers, particularly when lesions are located on the head and neck[4,7,9,22]. In contrast MAL-PDT has been shown to be less effective in the treatment of the pre-cancer AK when these lesions were located on the extremities/acral locations[6,13-16,18].

This study investigated the clinical outcome of MAL-PDT within two hundred (pre)cancerous skin lesions located at various anatomical locations (104 non-acral and 96 acral) as well as non-invasively monitoring the accumulation and destruction of the photosensitiser, PpIX. For each lesion included within the study a percentage change in fluorescence intensity following the three hour application of MAL was calculated and was considered to be representative of PpIX accumulation as previous studies have indicated the predominance of PpIX accumulation following topical ALA/MAL application[23]. Additionally the percentage change in fluorescence intensity following light irradiation was also calculated and this was considered to represent PpIX photobleaching as a result of the destruction of PpIX by singlet oxygen during the photodynamic reactions[1]. PpIX photobleaching has previously been demonstrated to correlate to cellular damage/death[24-27] and our studies have demonstrated that the percentage changes in fluorescence during light irradiation relate to clinical outcome[28].

This study considered the changes in fluorescence intensity within the lesions after the three hour application of MAL and the light irradiation stage of treatment (Fig. 1). Overall acral lesions were observed to undergo a significantly reduced percentage change following both the application of MAL ($P<0.05$) and the delivery of the total

light dose (P<0.001) indicating less PpIX was available for activation during irradiation, potentially reducing the effect of the treatment in these acral lesions. When the lesion types were considered individually only AK differed significantly in terms of the fluorescence changes witnessed with significantly reduced changes in PpIX fluorescence being observed within acral AK lesions following the application of MAL (P<0.01) as well as during irradiation (P<0.05). This may explain the poorer responses of acral AK lesions previously reported in the literature[13-18].

The direct comparison of PpIX accumulation and photobleaching of lesions located at acral and non-acral sites suggested that PpIX accumulation was observed to be significantly greater (P<0.001) than PpIX photobleaching when all 96 acral lesions were considered and when the 51 acral BD lesions were considered. This indicates that not all the PpIX produced in these lesions was utilised during the treatment, therefore potentially reducing treatment efficacy. In the non-acral group statistically greater accumulation was observed when the entire data set was considered (P<0.05), however no significant difference was observed when the lesions were considered individually. The non-acral lesions responded in a similar manner to our previous investigations which indicated similar levels of PpIX accumulation and photobleaching during MAL-PDT treatment (data not shown).

The complete clearance rates of the two hundred lesions considered here (Fig. 3) correspond with previous studies[13-18] indicating a reduction in treatment efficacy for lesions located in acral skin. Significantly reduced clearances were observed for acral lesions when the entire data set (P<0.01) and the AK lesions alone (P<0.05) were considered. No significant difference was observed in the complete clearances of acral and non-acral skin for BD and sBCC (P>0.1) (Fig. 3).

The clinical outcome of the lesion was determined by clinical evaluation at three months, as this is our standard clinical follow up protocol. However the results may have varied if the outcomes were determined at different time periods following treatment especially when considering longer term follow ups (e.g. several years). The current literature indicates that recurrence of a lesion can occur in regions previously considered clear several years following treatment[29]. However for the purposes of this study, whilst longer term follow-up may have been preferred, any recurrence is just as likely to occur in acral and non-acral skin and therefore we would predict that the complete clinical clearance rates at longer term follow ups should remain significant between acral and non-acral lesions. Furthermore, whilst histological analysis may be preferred to clinical evaluation, we wished to follow standard clinical PDT practice within our Department and preserve the excellent cosmetic outcomes achieved with PDT. The reviewing Consultant compared the treated area with the previous explanations within the notes, particularly comparing size and visual appearance of the area. The lesion was considered completely clear if no clinical signs were visible.

The fluorescence data provided an insight into the limited efficacy of MAL-PDT in the treatment of acral AK lesions. Firstly the significant reduction ($P<0.01$) in the level of PpIX attained following the application of the topical prodrug in acral skin was probably due to limited prodrug penetration through the stratum corneum into the diseased cells below. This reduction in PpIX accumulation may be a result of a thicker stratum corneum in acral skin [30], which has previously been demonstrated to weakly negatively correlate with PpIX fluorescence [31] or alternatively the increased number of pilosebaceous units of the head may improve PpIX accumulation within non-acral skin regions[32]. Secondly, acral AK lesions were observed to undergo a

statistically lower level ($P<0.05$) of PpIX photobleaching during light irradiation. Due to the established relationship between PpIX photobleaching and the subsequent cellular damage/clinical outcome observed[24,25,27,28], this reduction in PpIX photobleaching within acral AK lesions suggests lower levels of cellular damage/death and therefore an ultimately poorer clinical outcome as reported here. Therefore future work could endeavour to improve the accumulation of PpIX in AK lesions located in the extremities, perhaps utilising curettage[33], iron chelation[34-36] or manipulation of the haem biosynthesis pathway[37,38].

Interestingly, acral and non-acral BD and sBCC lesions were not noted to undergo significantly different clinical responses to MAL-PDT with the sample size employed here, although significantly further study would be required to determine this conclusively. BD lesions at acral sites did however undergo a significant reduction in the level of PpIX photobleaching when directly compared to the level of PpIX accumulated within the lesions, whilst BD lesions at non-acral sites underwent similar levels of accumulation and dissipation. This may be due to the predominance of BD lesions on the lower legs (72% of acral BD lesions within our data) where the oxygenation status of the skin tends to be greatly reduced when compared to the oxygenation status of facial skin[39,40], which would potentially limit the capacity of the photochemical reactions to produce singlet oxygen and other reactive oxygen species therefore diminishing the potential phototoxicity during light irradiation. The similarity between acral and non-acral sBCC in terms of PpIX accumulation, photobleaching and clinical response suggests that a thicker stratum corneum and limited pilosebaceous units on the head are not the key players in the significant difference. Therefore the differences observed within AK could be due to the more hyperkeratotic stratum corneum found within these lesion types in acral locations.

However, limited PpIX photobleaching was observed in acral BD lesions and whilst the hyperkeratotic stratum corneum may explain poor PpIX accumulation it is unlikely to prevent red light penetration and therefore future investigations need to consider the oxygenation status of the tissue to provide a greater insight into the discrepancy between acral and non-acral sites.

It is important to note that this study had several limitations. Firstly it was an observational study conducted in a clinical setting and therefore not powered in advance, the main reason behind this was to accurately follow standard dermatological PDT practice. Two hundred patients were considered resulting in a slightly unequal proportion of acral and non-acral lesions. However when the first fifty acral and the first fifty non-acral lesions were considered statistically significant differences remained in terms of PpIX accumulation and PpIX dissipation.

This study has investigated the clinical response of three types of skin lesions commonly treated with MAL-PDT and compared the outcomes of acral and non-acral lesions. As with previous investigations, a reduced clinical clearance rate was observed in acral lesions. For the first time this study monitored the changes in PpIX fluorescence throughout treatment, providing an insight into the reason behind the treatments' limited efficacy in the clearance of acrally located lesions, suggesting that PpIX accumulation and subsequent photobleaching are significantly reduced within lesions located at these sites. In AK lesions where previous studies have demonstrated a significant difference in clinical response between facial lesions and those located on other areas of the body, particularly the extremities, it would appear from this data that limited PpIX accumulation ultimately constrained the efficacy of the treatment and future work in this area should concentrate on resolving this issue.

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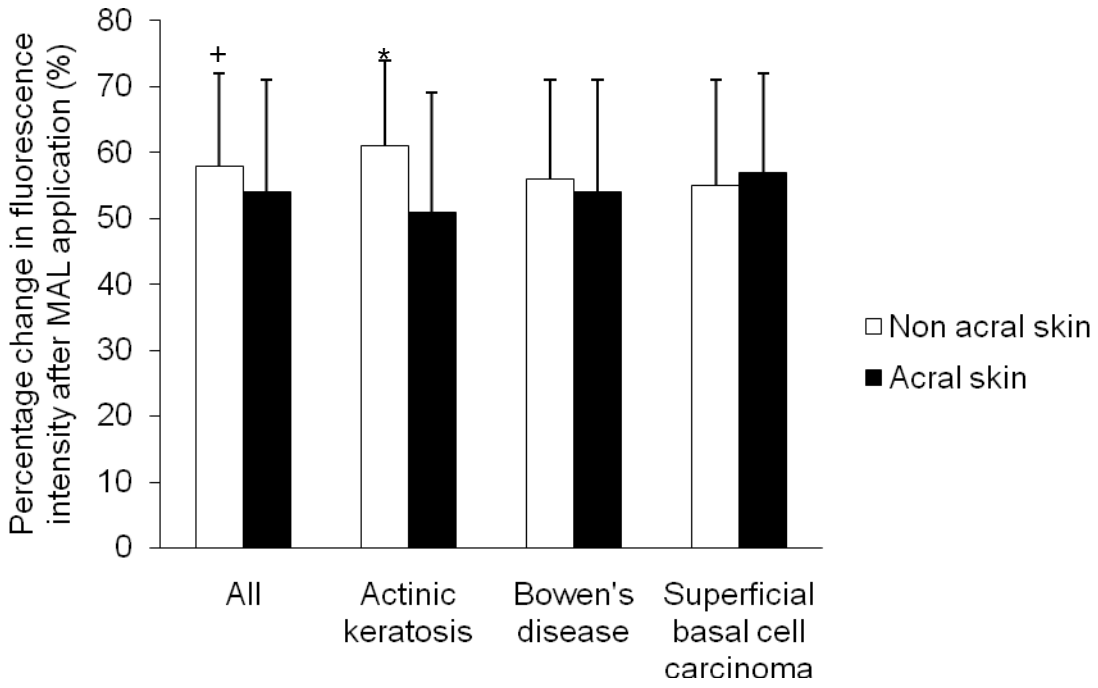
Fig 1. Bar charts indicating the percentage change in fluorescence within lesions following (a) the 3-h methylaminolaevulinate (MAL) application and (b) the light irradiation phase of treatment. +, * and Δ represent statistical significance at $P < 0{\cdot}05$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively, between acral and nonacral sites. The error bars represent the SD of the data.

Fig 2. Bar charts indicating the percentage change in fluorescence following the application of methylaminolaevulinate (MAL) and light irradiation for (a) acral and (b) nonacral sites. + and Δ represent statistical significance at $P < 0{\cdot}05$ and $P < 0{\cdot}001$, respectively, between acral and nonacral sites. The error bars represent the SD of the data. PDT, photodynamic therapy.

Fig 3. Bar chart indicating the percentage of acral and nonacral lesions undergoing complete clinical clearance 3 months after treatment. + and * represent significance between the different anatomical locations at $P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively.

a


Percentage change in fluorescence intensity after MAL application (%)
80
70
60
50
40
30
20
10
0
+
*
All
Actinic keratosis
Bowen's disease
Superficial basal cell carcinoma
Non acral skin
Acral skin

b

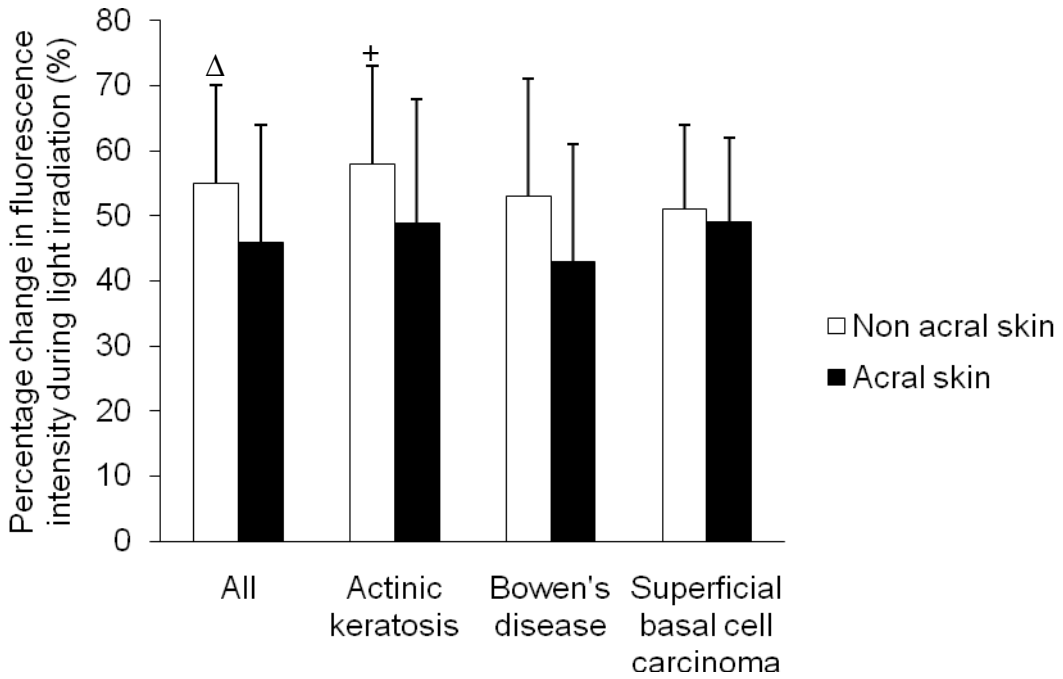
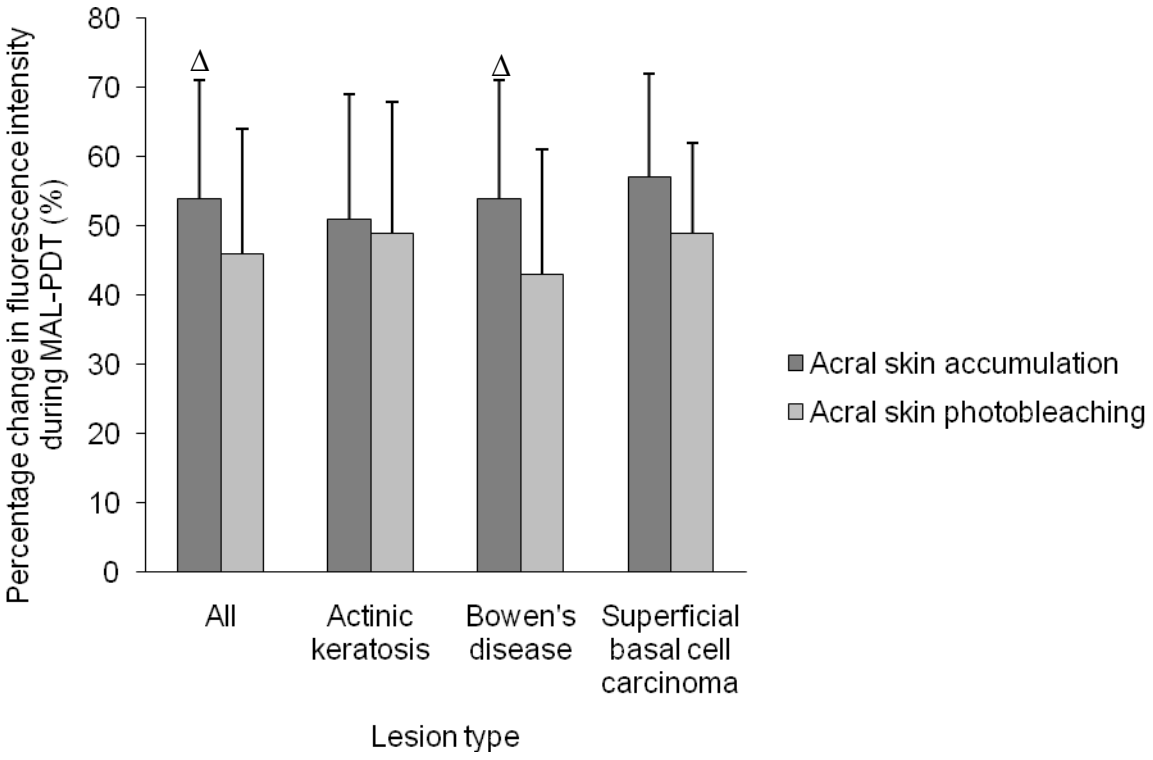

Percentage change in fluorescence intensity during light irradiation (%)
80
70
60
50
40
30
20
10
0
Δ
+
All
Actinic keratosis
Bowen's disease
Superficial basal cell carcinoma
Non acral skin
Acral skin

Figure 1

Acral PDT

a



b

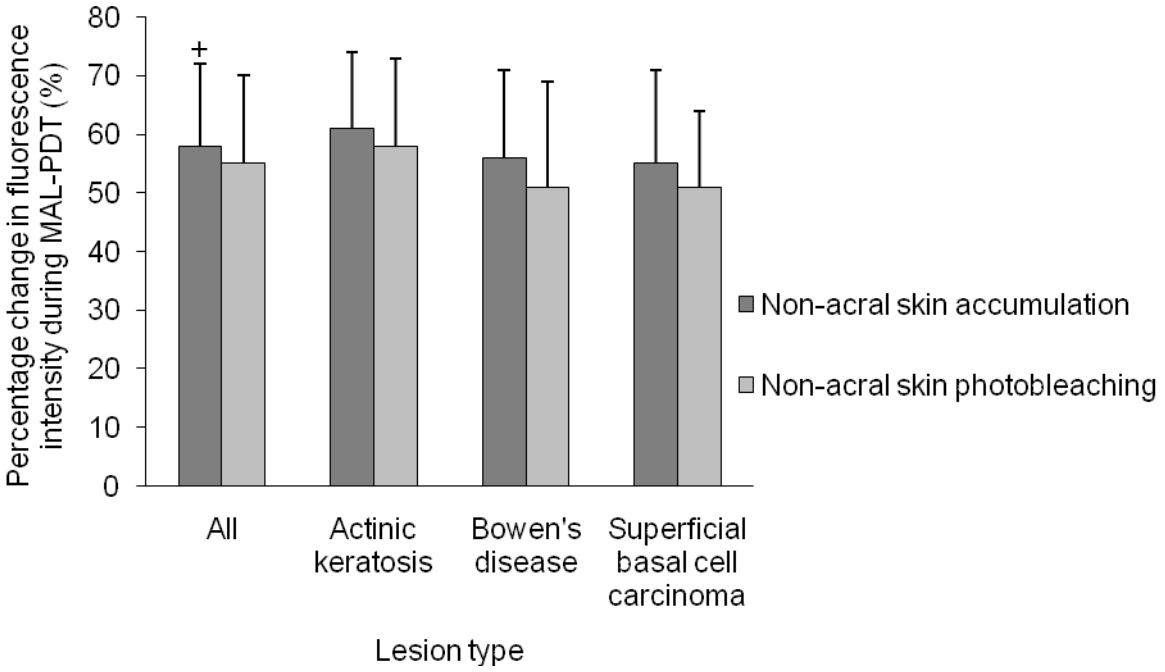


Figure 2

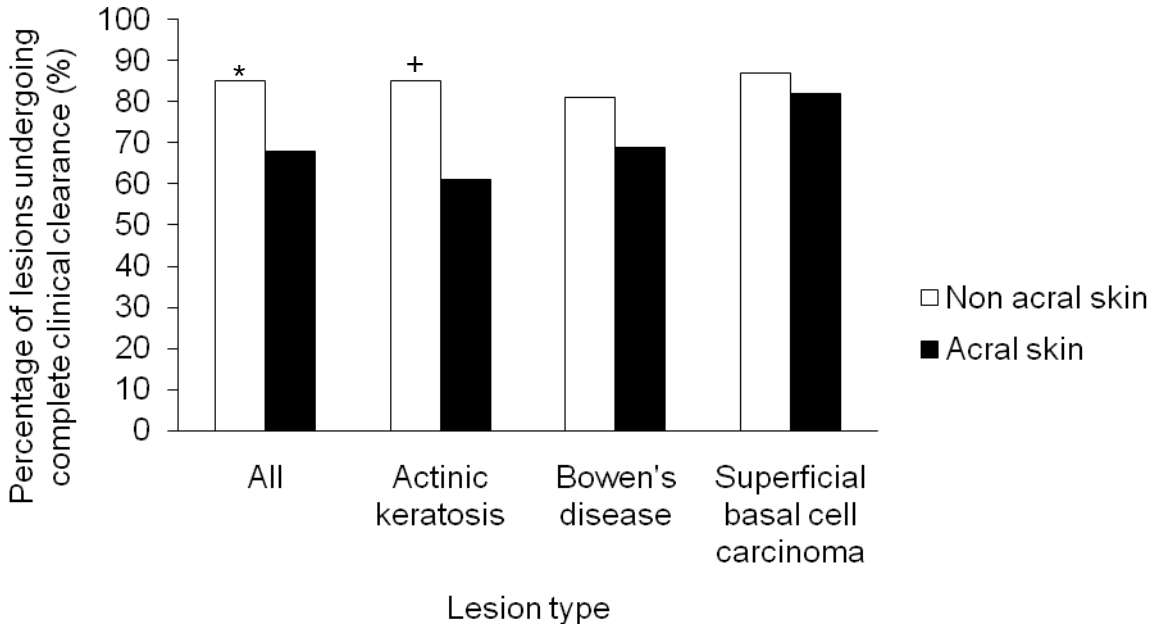

Percentage of lesions undergoing complete clinical clearance (%)
100
90
80
70
60
50
40
30
20
10
0
*
+
All
Actinic keratosis
Bowen's disease
Superficial basal cell carcinoma
Lesion type
Non acral skin
Acral skin

Figure 3