Correlation Between Macroscopic Fluorescence and Protoporphyrin IX Content in Psoriasis and Actinic Keratosis Following Application of Aminolevulinic Acid

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In fluorescence diagnosis with 5-aminolevulinic acid (ALA)-induced porphyrins (FDAP), protoporphyrin IX (PpIX) accumulation can be macroscopically visualized. Interpretation of these data is still problematic because of the low reproducibility of the procedure and poor understanding of the mechanisms involved in PpIX tumor selectivity. In this study, PpIX accumulation is investigated in patients with psoriasis and actinic keratosis (AK) following FDAP. For this purpose, desquamated lesional and non-lesional skin were incubated with 20% ALA ointment for 3 h, FDAP was performed, and highly fluorescing lesional skin and non-lesional skin were biopsied. In extracts from these biopsies, PpIX, protein, and dsDNA were quantified by spectrofluorometry. Digital images acquired with FDAP were analyzed using image analysis software. PpIX per biopsy in lesional skin in both psoriasis and AK was significantly higher than in non-lesional skin ($p < 0.05$). When corrected for epidermal involvement, only lesional psoriatic skin showed significantly higher PpIX levels than non-lesional skin. The PpIX-ratio lesional:non-lesional skin (mean(pmol per mL) $\pm$ SEM) was 4.12 ± 0.91 in psoriasis and 1.96 ± 0.24 in AK. In FDAP, the ratio of lesional: non-lesional skin was 1.77 ± 0.06 in psoriasis and 1.37 ± 0.07 in AK. Macroscopic fluorescence and PpIX content appeared to be well correlated ($r = 0.73$), thus making FDAP a good predictor of PpIX content.

Key words: actinic keratosis/5-ALA/fluorescence diagnosis/protoporphyrin IX/psoriasis
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Photodynamic therapy with 5-aminolevulinic acid (ALA) or its methylester (MAL) as a topical photosensitizer is gaining interest in an increasing number of dermatological centers. In this treatment the preferential accumulation of endogenous porphyrines, in particular protoporphyrin IX (PpIX), in lesional skin after incubation with ALA/MAL is used to treat various skin diseases (Kennedy *et al*, 1990; Foley, 2003). As PpIX has characteristic fluorescence properties, its preferential accumulation in certain cell types can also be used as a cellular marker. In fluorescence diagnosis with ALA-induced porphyrins (FDAP), lesional and non-lesional skin is irradiated with blue (Wood's) light after incubation of the skin with ALA. As PpIX shows red fluorescence when excited by blue light, PpIX accumulating cells can be visualized after irradiation with Wood's light (Fritsch *et al*, 2000). In cases where demarcation of lesional skin is clinically problematic or in dermatological screening of field cancerized skin, FDAP may provide additional information. Interpretation of these data is still problematic because of the low reproducibility of the procedure due to the numerous factors influencing fluorescence detection and poor understanding of the mechanisms involved in PpIX tumor selectivity.

Abbreviations: AK, actinic keratosis; ALA, 5-aminolevulinic acid; BCC, basal cell carcinoma; FDAP, fluorescence diagnosis with 5-aminolevulinic acid-induced porphyrins; NS, normal skin; PpIX, protoporphyrin-IX

Several different fluorescence detection techniques have been described by other authors studying PpIX fluorescence in human skin and mucosa (Andersson-Engels *et al*, 1995; Inaguma and Hashimoto, 1999; Wennberg *et al*, 1999; Leunig *et al*, 2001; Ericson *et al*, 2003b). Because of the different procedures that have been used, the results are difficult to compare. Wennberg *et al* reported a 50% correlation between macroscopic fluorescence imaging and histological mapping in 22 basal cell carcinoma (BCC) (Andersson-Engels *et al*, 1995; Wennberg *et al*, 1999). Lack of selectivity and great variation in microscopic intra- and inter-tumor fluorescence in BCC have been described by Martin *et al* (1995), whereas Peng *et al* (2001) found a highly selective and homogenous PpIX fluorescence in nodular BCC after 3 h incubation with MAL. Ericson *et al* (2003b) reported that macroscopic fluorescence levels 1.4 times above the mean normal skin fluorescence were associated with an abnormal condition in patients with facial BCC after 3–4 h incubation with ALA. The question remains, however, whether these high fluorescence values are generally associated with pathologic skin conditions. Macroscopic fluorescence intensity after incubation with ALA has been shown to differ in various skin diseases. Psoriasis has been reported to have the highest macroscopic fluorescence values but homogenicity appears to be a problem when lesions are not pretreated with a keratolytic agent (Fritsch *et al*, 2000). Macroscopic fluorescence intensity and intralesional PpIX content after ALA incubation have been

studied by Fritsch *et al* (1999). In this study, macroscopic fluorescence intensity was assessed by the human eye and given a value from 0 till 4 according to a fluorescence standard. With new digital imaging techniques, however, macroscopic fluorescence can be measured *in vivo* more accurately (Ackermann *et al*, 2000). In the same study, porphyrins were extracted, assessed by spectrofluorometry, and expressed per gram total protein. As PpIX is thought to be mainly formed in the epidermis, a more specific epidermal reference to relate PpIX content to would be more appropriate. As most PpIX is formed in the epidermis and most cellular nuclei are located intraepidermally, DNA content would be a suitable epidermal marker, The use of DNA as an epidermal reference has already been proven useful in several studies (van de Kerkhof *et al*, 1983a, b).

The aim of this study was to investigate PpIX accumulation in psoriasis and actinic keratosis (AK) and their surrounding clinically normal skin incubated with 20% ALA ointment for 3 h and to correlate these data to macroscopic fluorescence using a digital fluorescence imaging system. When this correlation is known, macroscopic fluorescence measured on the skin could be used as an estimate for actual PpIX content in the skin.

Results

Fluorescence spectrometry Fluorescence emission spectra ($\lambda_{excitation} = 408$ nm, slit $= 10$ nm) from ALA-treated skin samples showed maxima at around 630 nm with a smaller peak at around 700 nm. As PpIX is the predominant ALA-induced porphyrin metabolite (83.8%–92.6%) (Fritsch *et al*, 1999) and considering the emission maxima obtained from the ALA-treated samples to resemble the PpIX reference standard emission peak, one can assume these emission maxima at 630 nm to be predominantly caused by PpIX (Fritsch *et al*, 1999) (Fig 1). These maxima were most pronounced in ALA-treated skin samples from psoriasis, actinic keratoses, and their non-lesional skin, respectively. Basal levels of PpIX were below background fluorescence and therefore not accurately measurable.

Tissue PpIX content after ALA Lesional skin incubated with 20% ALA for 3 h showed higher total PpIX content than non-lesional skin in both psoriasis and AK. Lesional psoriatic skin showed higher PpIX levels compared with lesional skin in AK and non-lesional skin from psoriasis and AK, respectively (Fig 2, Table I).

Tissue dsDNA content In psoriasis and AK, lesional dsDNA content was higher compared with non-lesional skin in both diseases. Lesional psoriatic dsDNA content, however, was not statistically different from dsDNA content in lesional AK. When normal surrounding skin in AK and psoriasis was compared, normal surrounding skin in psoriasis appeared to contain more dsDNA than normal surrounding skin in AK (Fig 3, Table I).

Tissue PpIX content after ALA corrected for dsDNA - When correcting PpIX content for dsDNA content, psoriasis again showed statistically significantly higher levels of PpIX compared with normal psoriatic skin. In AK, however, PpIX levels in lesional skin did not differ from PpIX levels in non-lesional skin. PpIX content in lesional psoriatic skin was also higher than in lesional skin in AK (Fig 4, Table I).

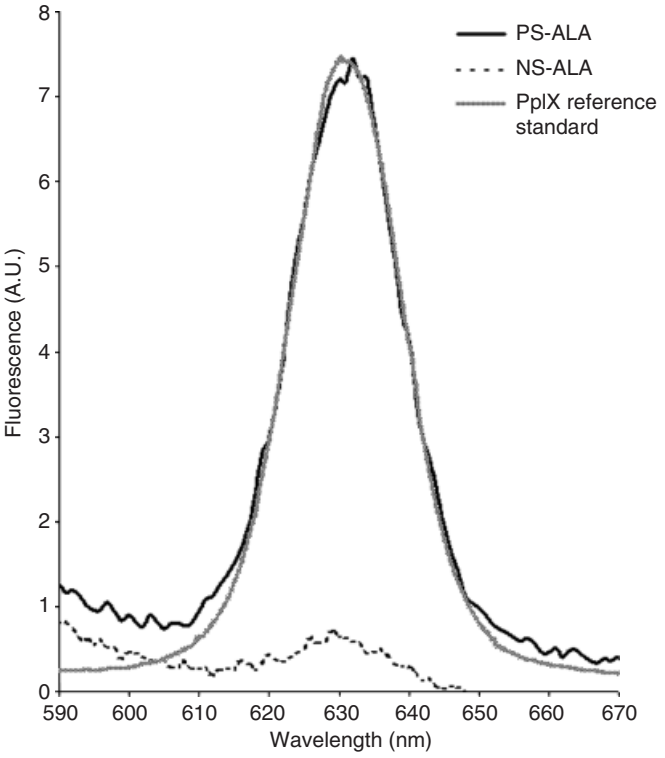

Figure 1
Emission spectrum of extracts from 20% 5-aminolevulinic acid (ALA) (3 h)-incubated psoriatic skin samples and protoporphyrin-IX (PpIX) reference standard. PS-ALA: psoriatic lesion after 20% ALA (3 h), NS-ALA: normal skin in psoriasis after 20% ALA (3 h). The emission spectra ($\lambda_{excitation} = 408$ nm) of 20% ALA (3 h) incubated psoriatic skin samples and PpIX reference standard are very similar.

Photobleaching studies The PpIX emission peak (630 nm) declined following irradiation with 415 ± 5 nm at 40 mW per cm^2 ($p < 0.001$) (Fig 5). With increasing fluence, a second

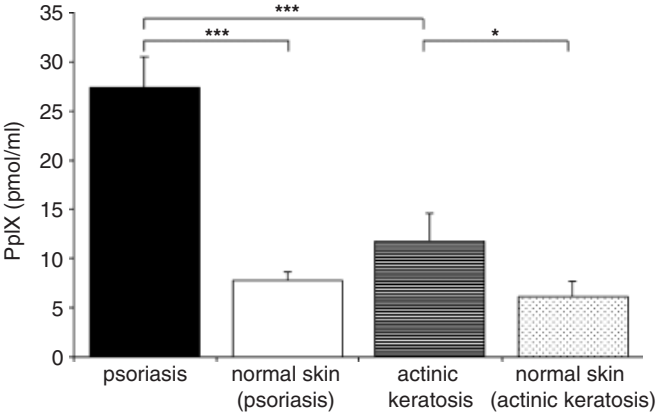

Figure 2
Tissue protoporphyrin-IX (PpIX) content after 20% 5-aminolevulinic acid (3 h). From 4 mm punch biopsies, PpIX was extracted and quantified by spectrofluorometry. $^{*}p < 0.05$, $^{***}p < 0.001$. Error bars denote standard errors of the mean.

Table I. Summary of results

Skin type	PpIX (pmol per mL)	dsDNA (μg per mL)	PpIX/dsDNA (pmol PpIX per μg dsDNA)	PpIX ratio after ALA	Macroscopic fluorescence ratio	
					Before ALA	After ALA
Psoriasis	27.4 ± 3.1	1.7 ± 0.2	16.3 ± 2.4	4.1 ± 0.9	0.90 ± 0.03	1.77 ± 0.06
Normal skin (psoriasis)	7.80 ± 0.81	1.1 ± 0.2	7.5 ± 1.5			
Actinic keratosis	11.8 ± 2.6	1.5 ± 0.3	7.7 ± 1.4	2.0 ± 0.2	0.98 ± 0.03	1.37 ± 0.07
Normal skin (actinic keratosis)	6.2 ± 1.9	0.58 ± 0.09	7.6 ± 1.9			

Values are denoted ± standard error of the mean.
PpIX, protoporphyrin IX; ALA, 5-aminolevulinic acid.

peak arose at 670 nm, which is thought to be the photoproduct of PpIX (Ppp) (Ericson *et al*, 2003a). The decline in the PpIX emission peak was most pronounced in pure PpIX solutions obtained from the PpIX reference standard kit compared with PpIX-enriched normal skin extracts (Fig 6). Two-way ANOVA also revealed a significant difference between the PpIX-enriched and the pure PpIX solutions ($p<0.01$). This indicates a slower photobleaching of PpIX-enriched normal skin extracts and is also demonstrated by a greater fluence required to halve the initial PpIX content (Fig 6). Considering these half-lives to be relatively high, possible photobleaching effects due to ambient light exposure during the extraction procedure can therefore be neglected.

Image analysis In both psoriasis and actinic keratoses, incubation with 20% ALA for 3 h induced a statistically significant increase in lesional skin: normal skin fluorescence ratio. In psoriasis, this increase was more pronounced than in AK (Table I, Figs 7 and 8).

Correlation between PpIX content and macroscopic fluorescence When all corrected fluorescence data from the Dyaderm system were compared with their accompanying PpIX values, a positive correlation was found as seen in Fig 9. Correlation analysis revealed a correlation coefficient of $r=0.73$. When correlation curves for AK and psoriasis were analyzed separately, the correlation coefficient for AK appeared to be somewhat higher compared with psoriasis ($r=0.79$ *vs* 0.69, respectively).

Discussion and Conclusions

Treatment with ALA ointment under an occlusive dressing for 3 h of lesional and non-lesional skin in AK and psoriasis induces an increase in biochemical tissue porphyrin content. This increase was significantly higher in lesional skin compared with non-lesional skin and was most pronounced in psoriasis when compared with AK, which is in accordance with other studies (Fritsch *et al*, 1999). In our study, porphyrin content was quantified using a PpIX reference standard. This can be justified as PpIX is the predominant porphyrin comprising about 75% (Fritsch *et al*, 1999) of total ALA-induced porphyrins after 3 h incubation. This also becomes clear when the emission spectra obtained from ALA-treated skin and the PpIX reference standard are compared (Fig 1). Although the composition of porphyrin metabolites is known to change in time following ALA application, porphyrin patterns, however, do not significantly differ between various skin diseases, including normal skin, BCC, squamous cell carcinoma (SCC), and psoriasis (Fritsch *et al*, 1999). Furthermore, one can discuss whether these exact porphyrin compositions are clinically relevant when

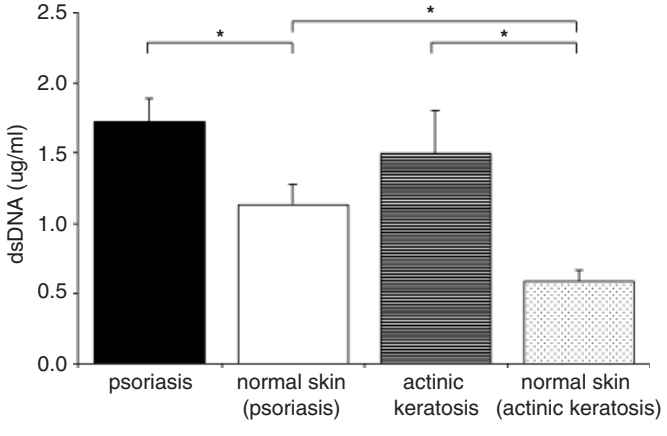

Figure 3
Tissue dsDNA content. From 4 mm punch biopsies, dsDNA was extracted and quantified by spectrofluorometry. *$p<0.05$. Error bars denote standard errors of the mean.

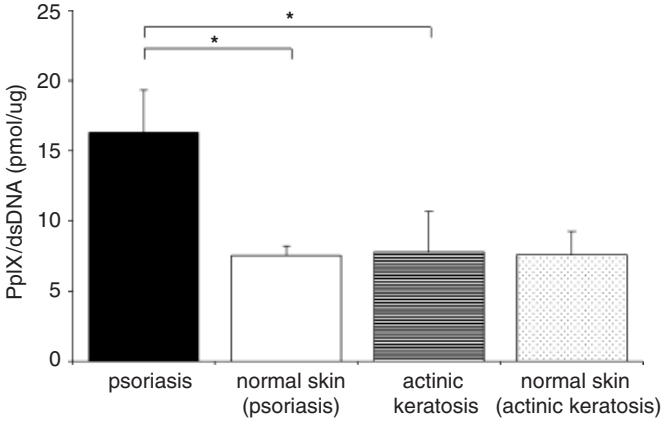

Figure 4
Tissue protoporphyrin-IX (PpIX) content after 20% 5-aminolevulinic acid (3 h) corrected for dsDNA. PpIX values were divided by their accompanying dsDNA values. *$p<0.05$. Error bars denote standard errors of the mean.

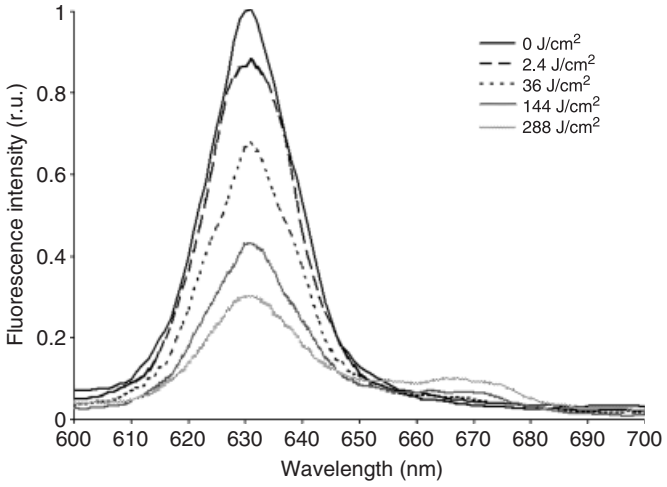

Figure 5
Emission spectra of irradiated protoporphyrin-IX-enriched normal skin sample solutions. Excitation wavelength was set to 408 nm. Samples were exposed to 0, 2.4, 36, 144, and 288 J per cm^2 using the Waldmann Omnilux Blue (415 $\pm$ 5 nm, 40 mW per cm^2). Note that the 630 nm emission maximum declines and a new maximum arises at 670 nm following higher fluences. Fluorescence intensity was measured in relative units.

performing FDAP as changes in "porphyrin-like fluorescence" (630 nm) are measured.

In our study, porphyrins were measured after 3 h ALA application. This timepoint was found to have optimal tumor: normal skin contrast ratio when performing FDAP according to Ericson *et al* (2003b). For higher lesional fluorescence values, a longer incubation period up to 6 h is needed, but surrounding (normal) skin fluorescence also increases, thus reducing fluorescence contrast ratio. Tumor: normal skin ratios of total biochemical tissue porphyrin content peak between 1 and 4 h of ALA incubation in BCC and SCC; in psoriasis, however, this ratio is optimal after 6 h ALA incubation (Fritsch *et al*, 1999).

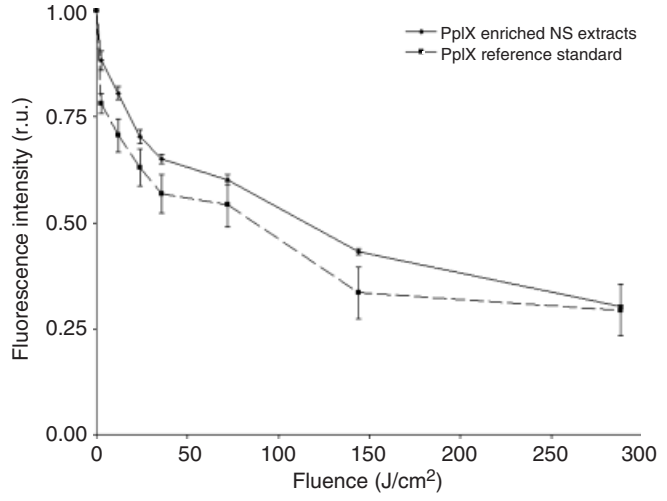

Figure 6
Photobleaching of protoporphyrin-IX (PpIX)-enriched normal skin extracts and PpIX solutions. PpIX-enriched normal skin extracts appeared to be less susceptible to photobleaching compared with pure PpIX solutions ($p<0.01$). Fluorescence intensity was measured in relative units. NS, normal skin. Error bars denote standard errors of the mean.

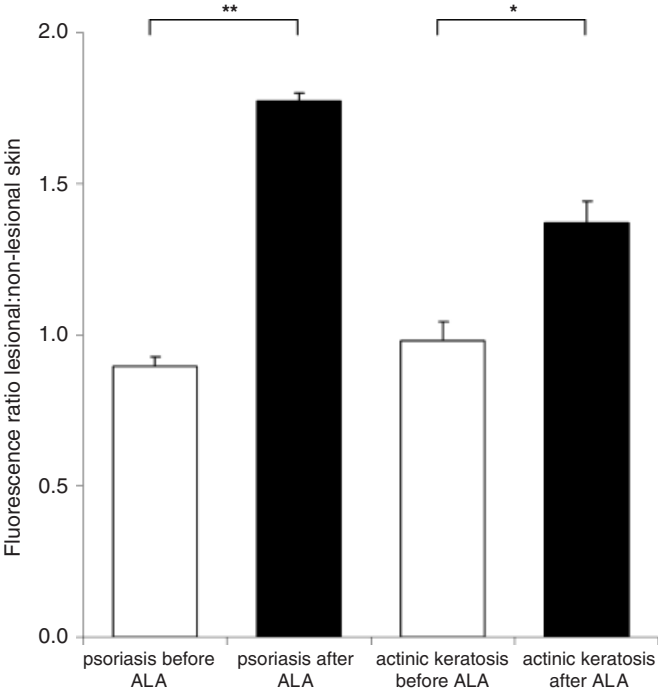

Figure 7
Macroscopic fluorescence *in vivo* (FDAP) ratio lesional skin: non-lesional skin in psoriasis and actinic keratosis before and after incubation with 20% 5-aminolevulinic acid ointment for 3 h. $^*p<0.05$, $^{**}p<0.01$. Error bars denote standard errors of the mean.

As PpIX is formed and accumulated mainly in the epidermis, PpIX data were also corrected for epidermal involvement using dsDNA as an epidermal reference. After this correction, in AK, no statistically significant increase in PpIX per μg dsDNA was seen any longer after ALA treatment. But, PpIX per μg dsDNA in psoriasis was still significantly higher after ALA treatment. These findings suggest different mechanisms being mainly responsible for increased PpIX formation in psoriasis and AK. In psoriasis, increased PpIX formation per cell may play a more important role than in AK. Various mechanisms have been proposed for this by other investigators. Alterations in the heme-biosynthethic pathway in hyperproliferative tissue are thought to play an important role in preferential PpIX accumulation including increased porphobilinogen deaminase (PBGD) and/or decreased ferrochelatase activity (Hinnen *et al*, 1998; Krieg *et al*, 2002). An increased uptake of ALA by pathologic tissue may also be responsible for increased PpIX formation, for example due to a defective skin barrier function, which has been suggested by Martin *et al* (1995) in BCC. In psoriasis this may also be the case as defects in stratum corneum structure have been described (Ghadially *et al*, 1996).

Our data show no statistically significant differences in PpIX content per cell in lesional AK compared with surrounding non-lesional AK. This suggests the number of cells producing PpIX to be the most important factor for macroscopic skin fluorescence *in vivo* as seen with FDAP. This is in parallel with our findings that dsDNA content is significantly increased in lesional AK compared with non-lesional surrounding skin. Although it can not be excluded that in AK alterations in the heme-biosynthetic pathway or increased

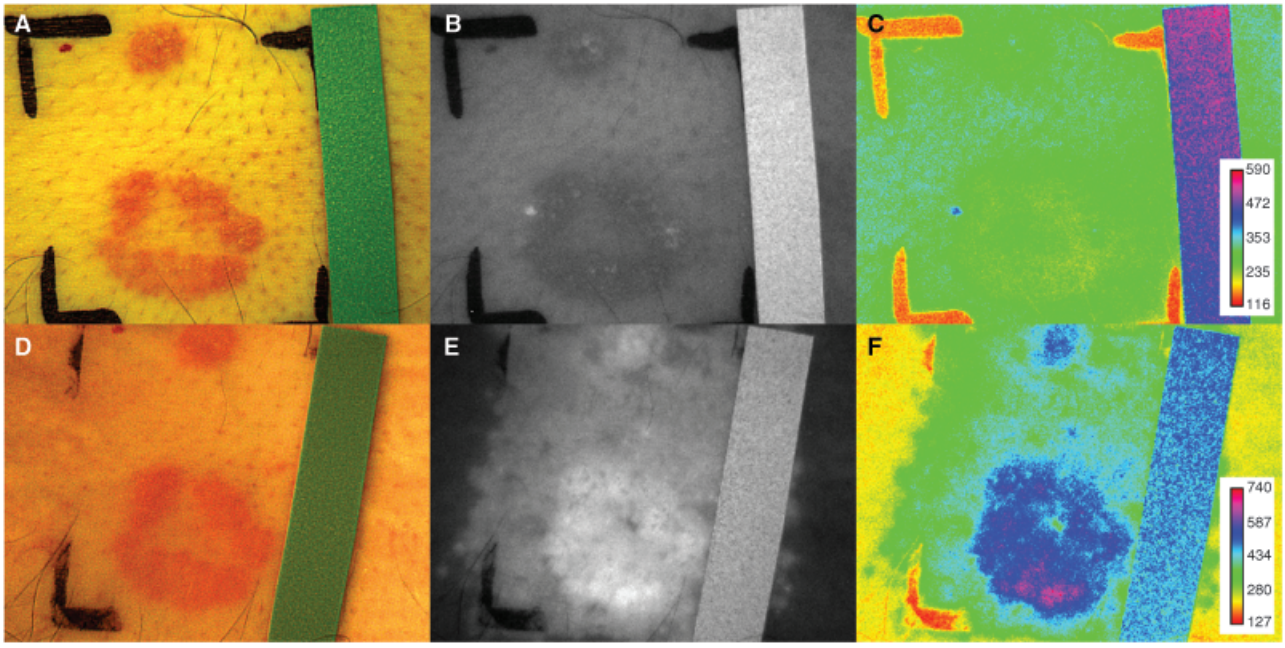

Figure 8
Normal, fluorescence, and pseudo-color images from psoriatic skin *in vivo*. Before (*A–C*) and after incubation with 20% 5-aminolevulinic acid (ALA) ointment for 3 h (*D–F*). Incubation with 20% ALA (3 h) ointment induced a remarkable increase in red fluorescence, which was more pronounced in lesional skin.

uptake of ALA may also play a role, this could not be demonstrated in our study. Additional studies analyzing PpIX accumulation in cell cultured systems of AK and psoriasis could provide further information concerning this issue.

Initially, total protein was measured as an epidermal reference value but this did not appear to be an appropriate reference eventually: protein levels in the biopsies between lesional and non-lesional skin in psoriasis did not differ much from each other with a large standard deviation, which could be explained by the fact that the amount of dermis in the biopsies was not standardized. As lesional skin in psoriasis is known to have higher epidermal protein levels because of the higher proliferation rate, this suggests the dermis to have a substantial contribution to total protein content, thus overshadowing differences in epidermal protein content (van Erp and van de Kerkhof, 1988). DsDNA quantification revealed evident differences in the four skin types and appeared to be a good marker for the amount of epidermal keratinocytes. In hyperproliferative skin conditions, total DNA content is commonly increased due to increased DNA synthesis, which can be a disturbing factor when a comparison is made with normoproliferative skin. In a study of Bauer *et al* (1981), the percentage of cells in the S-phase in psoriasis appeared to be increased compared with normal skin (9.7% *vs* 2.7%). In non-involved psoriatic skin, this percentage of cells in S-phase was 3.8%. To the best of our knowledge, in AK, these exact percentages are not known but when proliferation indices (PI) of psoriasis and AK were compared (using Ki-67 expression) in a study of Caldwell *et al* (1997) AK appeared to have a PI that is 1.26 times the PI in psoriasis. This means that in our samples, dsDNA values as a marker of the total number of epidermal keratinocytes could be overestimated. When correcting our dsDNA data accordingly, lesional psoriatic skin and AK data had to be decreased by 7.25% and 9.14%, respectively, and their non-lesional skin to a lesser extent. These corrections, however, did not have a significant effect on our overall data.

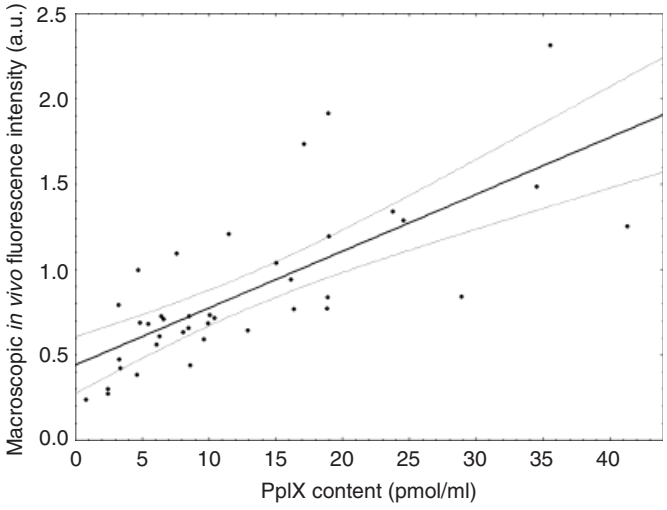

Figure 9
Correlation between protoporphyrin-IX content in the skin and macroscopic fluorescence at the skin. $r = 0.73$, $p < 0.001$. Dotted lines indicate 95% confidence intervals.

Our results show a positive relation between macroscopic fluorescence as measured with our digital imaging system and biochemical tissue PpIX content. Although other authors have described *in vivo* measuring of PpIX, macro-, and microscopic fluorescence imaging after ALA treatment or combinations, to the best of knowledge, no one has linked digital macroscopic imaging directly to biochemical tissue porphyrin content (Martin *et al*, 1995;

Wennberg *et al*, 1999; Ericson *et al*, 2003b). Especially in the lower fluorescence range up to 1.0 fluorescence intensity, in which most actinic keratotic lesions lie, this correlation was particularly strong (Fig 9). When psoriatic and AK values were analyzed separately, the slope of the correlation curve in psoriasis showed a more vertical course (not shown). This might be explained by the limited penetration into the deeper skin layers of the blue excitation light in our imaging system. PpIX deep in the epidermis, which is still detectable by spectrofluorometric analysis might not be fully excited and detected by FDAP. This is one of the main limitations of FDAP and emphasizes the need for adequate desquamation before performing FDAP. The question remains to what extent PpIX fluorescence is influenced by a non-standardized desquamation procedure, e.g., curretage, as external injury to the skin may induce false-positive fluorescence by a disrupted stratum corneum and/or increased wound repair theoretically. As basal PpIX levels in psoriatic stratum corneum are increased compared with other skin diseases (Bissonnette *et al*, 1998) inadequate desquamation can also lead to false-positive fluorescence values when performing FDAP. In our study protocol, desquamation was achieved by the use of 10% salicylic acid in vaseline twice daily 1 wk prior to the day of investigation, thus preventing any interference of scales.

When properly standardized as performed with our digital imaging system, FDAP appeared to be a good measure for PpIX content in the skin. This can be helpful in the clinical setting when PpIX accumulation is used for example as a guiding tool in taking biopsies in field cancerized skin, in demarcation of (non-melanoma) skin tumors or to evaluate the treatment efficacy in non-melanoma skin cancer. Further studies are, however, needed to scrutinize the mechanisms involved in preferential PpIX accumulation and the factors influencing FDAP, so that fluorescence values can be adequately interpreted. These data and a standardized treatment protocol are needed to assess the sensitivity and specificity of the procedure so that FDAP can become a more reliable diagnostic tool.

Material and Methods

Study population This study was approved by the local ethics committee and was conducted according to the Declaration of Helsinki Principles. After given their written informed consent, 11 patients with plaque-type psoriasis and 11 patients with AK were included in this study. All patients had a long history of psoriasis or AK that was histologically confirmed. The studied lesions were all located on the extremities. Lesional skin was pretreated with 10% salicylic acid in vaseline for 1 wk prior to the day of ALA application to remove any scales that could interfere with ALA uptake and/or light penetration. Prior to ALA application, lesions were photographed with the DyaDerm digital imaging system (Biocam GmbH, Regensburg, Germany) after desinfection with 80% denaturated alcohol vol/vol. Afterward, 20% 5-ALA ointment (Medac Gmbh, Wedel, Germany) was applied to an area of 5×8 cm including lesional and non-lesional skin. Over this area, Tegaderm (3 M Pharmaceuticals, Zoeterwoude, the Netherlands) and aluminum foil were applied as an occlusive dressing and to protect against unwanted light effects. Three hours later, the ALA ointment was removed, again photographs were taken with the digital imaging system, and 4 mm punch biopsies were taken from non-lesional skin and fluorescing lesional skin.

Image-capturing procedure The DyaDerm digital imaging system consists of a flash light (Xenon light source with a custom bandpass filter (370–440 nm)) and a 12-bit Sony CCD camera combined in one adjustable arm coupled to a Pentium IV computer equipped with custom-made image capturing software (Dyaderm Pro v1.4, Biocam GmbH). The flash light emits 7 light pulses per second to the skin, which are recorded by the CCD camera (exposure time 100 μs) equipped with a special Schott GG 455 longpass filter to filter out the excitation light. As PpIX fluorescence emission consists of light in the red spectrum, the red pixels of the CCD camera (spectral sensitivity at 630 nm between 85% and 90%) were used to generate a fluorescence image. In this way, a normal colored image and a fluorescence image were processed in real time. Because of the short exposition time to the excitation light, photobleaching of PpIX was minimalized in this way. To correct for different lighting environments between pictures, a fluorescence reference standard (Maccal 8044, 738-00, Multifoil, Utrecht, the Netherlands) was included on every image. Images were recorded in 16-bit grayscale TIFF format.

Skin biopsy procedure The 4 mm punch biopsies were taken in a darkened room under local anesthesia with 1% xylocain–adrenalin from highly fluorescing lesional and non-lesional skin after 3 h incubation with 20% ALA ointment. Highly fluorescing areas from lesional skin were located with the digital imaging system and marked to identify the site of biopsy. Biopsies from non-lesional (surrounding) skin were taken out of an area representing mean non-lesional skin fluorescence. Here again, the site of the biopsy was carefully selected and marked with the digital imaging system so that macroscopic fluorescence at the site of biopsy and PpIX per biopsy matched. Samples were directly frozen into liquid nitrogen afterward. To minimize possible PpIX stability problems, tissue extraction and spectrofluorometrical analysis of PpIX was performed on the same day.

Tissue extraction procedure The 4 mm punch biopsies were trimmed to remove a large part of the dermis, frozen in liquid nitrogen, and homogenized at 2,000 r.p.m. with the Microdismembrator U (B. Braun Biotech International, Melsungen, Germany) for 2 min. Chloroform:methanol (2:1 vol/vol, Merck KgaA, Darmstadt, Germany) mixture was used as an extraction medium. Mixtures were centrifuged for 15 min at 15,000 r.p.m. (23,140 $\times$ *g*), and the supernatant was collected for further analysis. A second extraction cycle was performed by adding the same amount of chloroform:methanol (2:1 vol/vol) mixture to the pellet. This mixture was vortexed for at least 5 min until a new suspension was formed and centrifuged for 15 min at 15,000 r.p.m. (23,140 $\times$ *g*). Subsequently, the supernatant was collected and added to the supernatant from the first extraction cycle. The PpIX extraction procedure was identical to the procedure used by Inaguma and Hashimoto (1999) apart from our homogenization technique. For dsDNA and protein extraction out of the remaining pellet, phosphate-buffered saline (B. Braun, Melsungen, Germany) was used as an extraction medium with a similar extraction protocol as decribed above. All these steps were performed in a dark laboratory environment taking ultimate care to prevent exposure of the specimens to light.

Spectrofluorometric analysis In the supernatant, the PpIX content was assessed by fluorescence spectrophotometry (Shimadzu RF-5001 PC, Den Bosch, the Netherlands); excitation was set to 408 nm (slit 10 nm) and emission was recorded at 630 nm (slit 10 nm). An emission spectrum (excitation wavelength set at 408 nm) was also recorded in the range from 450 to 800 nm. For quantification, a PpIX reference standard was used (Porphyrin Products Inc., Logan, Utah). Chloroform:methanol (2:1 vol/vol) mixture was used as a diluent. For protein quantification and dsDNA quantification, the Nano Orange protein quantitation kit (Molecular Probes, Eugene, Oregon) and Picogreen dsDNA quantitation kit (Molecular Probes) were used, respectively.

Photobleaching studies and recovery To evaluate possible PpIX loss due to photobleaching during the extraction protocol and to assess the internal validity, several photobleaching studies were performed. In these studies, four different PpIX solutions from the PpIX calibration curve (84, 33, 12 and 5 pmol per mL PpIX) and four normal skin extracts with addition of four different PpIX solutions (104, 54, 26, 13 and 3 pmol per mL PpIX) were exposed to various fluences (0, 2.4, 36, 144 and 288 J per cm^2) using the Waldmann Omnilux Blue (415 ± 5 nm, 40 mW per cm^2, Waldmann Medische Techniek B.V., Tiel, the Netherlands). These PpIX-enriched normal skin extracts were produced by mixing 100 μL of PpIX solution with 900 μL of the normal skin extract, resulting in comparable PpIX concentrations as the four pure PpIX solutions. At each fluence, an emission spectrum ($\lambda_{excitation} = 408$ nm, slit $= 10$ nm) was recorded. The PpIX-enriched concentrations were chosen in the same range as our measurements of PpIX in ALA-treated skin and were in the reliable range of our calibration curve. Normal skin was obtained from excess skin from patients undergoing abdominal plastic surgery.

Image analysis The 16-bit grayscale TIFF fluorescence images were imported in NIH ImageJ software (http://rsb.info.nih.gov/ij/). Because the Xenon light source used for excitation has the highest intensity in the center of the illuminated area, shading correction was performed by means of the following algorithm:

$$S = I/C \text{ with } C = B/B_{Imax}$$

where B is the blank image (image from a white homogenous background recorded with the Dyaderm system), B_{Imax} the highest intensity of blank image, I the (uncorrected) image, C the normalized shading image, and S the shading corrected image.

Subsequently, the mean pixel value of the biopsy sites and fluorescence standard were measured and recorded. To correct for different lighting conditions between the pictures, all measured values were divided by the value of the accompanying fluorescence reference standard.

Statistical analysis For analysis of PpIX content, dsDNA values, and macroscopic fluorescence *in vivo* a paired two-tailed Student's *t*-test was used. A p-value < 0.05 was regarded as statistically significant. Correlation analysis of PpIX content and macroscopic fluorescence *in vivo* was performed using Pearson's correlation analysis. All statistical calculations were performed using Statistica 6.0 software (Statsoft Inc., http://www.statsoft.com).

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