

Quantitative approach to skin field cancerization using a nanoencapsulated photodynamic therapy agent: a pilot study

Simone K Passos^{1,2}
Paulo EN de Souza³
Priscila KP Soares^{1,3}
Danglades RM Eid^{1,2}
Fernando L Primo⁴
Antonio Cláudio Tedesco⁴
Zulmira GM Lacava¹
Paulo C Morais^{3,5}

¹University of Brasília, Institute of Biological Sciences, DF, Brazil;

²Foundation for Teaching and Research on Health Sciences, Brasília, DF, Brazil; ³University of Brasília, Institute of Physics, Brasília, DF, Brazil;

⁴Department of Chemistry, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, Laboratory of Photobiology and Photomedicine, University of São Paulo, Ribeirão Preto, São Paulo, Brazil; ⁵Department of Control Science and Engineering, Hua-Zhong University of Science and Technology, Wuhan, People's Republic of China

Background: This paper introduces a new nanoformulation of 5-aminolevulinic acid (nano-ALA) as well as a novel quantitative approach towards evaluating field cancerization for actinic keratosis and/or skin photodamage. In this pilot study, we evaluated field cancerization using nano-ALA and methyl aminolevulinate (MAL), the latter being commercialized as Metvix®.

Methods and results: Photodynamic therapy was used for the treatment of patients with selected skin lesions, whereas the fluorescence of the corresponding photosensitizer was used to evaluate the time evolution of field cancerization in a quantitative way. Field cancerization was quantified using newly developed color image segmentation software. Using photodynamic therapy as the precancer skin treatment and the approach introduced herein for evaluation of fluorescent area, we found that the half-life of field cancerization reduction was 43.3 days and 34.3 days for nano-ALA and MAL, respectively. We also found that nano-ALA targeted about 45% more skin lesion areas than MAL. Further, we found the mean reduction in area of skin field cancerization was about 10% greater for nano-ALA than for MAL.

Conclusion: Although preliminary, our findings indicate that the efficacy of nano-ALA in treating skin field cancerization is higher than that of MAL.

Keywords: photodynamic therapy (PDT), nanoemulsion, 5-aminolevulinic acid, actinic keratosis, field cancerization, skin cancer

Introduction

Nowadays, the term “field cancerization” is used broadly to “mean the process whereby cells in a particular tissue or organ are transformed, such that genetically altered but histologically normal-appearing cells predate the development of neoplastic disease or coexist with malignant cells.” Ultimately, field cancerization is related to cells with cancer-associated genetic alterations but without invasive growth.¹ Field cancerization has been reported on almost all epithelial surfaces, in particular on the skin, which is the organ most exposed to environmental carcinogens, such as ultraviolet radiation,^{1,2} and also to other skin disorders that can evolve to a skin cancer.³ The preneoplastic phase in skin likely reflects accumulation of mutations¹ that form the basis for the carcinogenesis process.^{1,4-6} The literature emphasizes that if a tumor is excised from a predisposed epidermis, recurrence may occur by neoplastic conversion of residual parts of field cancerization.⁶ Furthermore, it has been widely reported that after a first tumor occurrence, the chance of recurrence is statistically enhanced.⁷

The most common skin lesion caused by ultraviolet B radiation is actinic keratosis, which is considered the precursor lesion of squamous cell carcinoma. Actinic keratosis

Correspondence: Zulmira GM Lacava
Institute of Biological Sciences, University of Brasília, 70-910-900 Brazil
Tel +55 61 3107 3087
Fax +55 61 3107 3087
Email zulmira@unb.br

and invasive squamous cell carcinoma share histopathological features, and both present genetic tumor markers and p53 mutation.⁸ However, there is accumulating evidence that actinic keratosis and squamous cell carcinoma are part of a broad spectrum, running from sun-damaged skin to squamous cell carcinoma, with both demonstrating alterations in the p53 gene, the signature of early skin carcinogenesis. Studies reveal a direct correlation between abnormal gene expression and progression from normal skin to photodamaged skin and from actinic keratosis to squamous cell carcinoma.^{9,10} Actinic keratosis has been classified as early squamous cell carcinoma in situ type I, II, and III.^{11–13} An estimated 12%–13% of patients with untreated actinic keratosis may develop invasive squamous cell carcinoma.¹⁴ Organ transplant recipients are particularly and severely affected by skin-related field cancerization; within 5 years of transplant, about 40% of organ transplant recipients develop actinic keratosis and have a 40–250-fold increase in occurrence of squamous cell carcinoma, with a 10-fold increase in mortality due to squamous cell carcinoma.¹⁵ Current international guidelines recommend treatment of actinic keratoses to avoid the potential for their development into squamous cell carcinomas.^{14,16–18} Exposure to ultraviolet B radiation, which indeed triggers molecular changes, precedes the appearance of actinic keratosis, and these lesions can be diagnosed at the subclinical stage using molecular techniques, although these methods would be difficult to implement on a large scale for routine screening. However, the fluorescence recorded from photosensitizing agents used in photodynamic therapy (PDT) can be used qualitatively to assess for the presence of field cancerization in the skin,¹⁹ thus offering a great window of opportunity in the routine hospital setting for the diagnosis and treatment of field cancerization in the preclinical phase. Nevertheless, a protocol for quantitative evaluation of the extent of field cancerization using the fluorescence of photosensitizers has not been devised as yet. Difficulty in assessing such information persists, leading to naïve control of efficacy of the cancer therapy and prevention approaches used.

PDT has been used worldwide for treatment of field cancerization, being very effective for actinic keratosis, while employing 5-aminolevulinic acid (ALA) or its derivative methyl aminolevulinate (MAL) as photosensitizing precursors.^{8,19–22} In short, PDT is based on activation of a photosensitizing precursor by visible light to produce cytotoxic oxygen species in the presence of oxygen contained in cells or tissues, that ultimately promote cell death.²² For treatment of actinic keratosis, the photosensitizing precursor is applied over the skin lesion

and, after a period of occlusion, the photosensitizing precursor (ie, ALA or MAL) is converted into photoactivatable porphyrins, in particular protoporphyrin IX, with preferential accumulation in neoplastic or dysplastic tissues.²³ After the period of occlusion, application of visible light triggers production of activated (cytotoxic) oxygen species, thereby leading to cell damage and consequent cell death. The safety and efficacy of topical ALA and MAL for PDT have been fully demonstrated in the treatment of actinic keratoses.^{18,23–30} Nevertheless, the instability of ALA in aqueous formulation and its low lipid solubility, the latter limiting the ability to penetrate through skin or cell membranes, have been identified as the main drawback of this photosensitizing precursor, thus restricting its use in PDT to superficial disease.³¹ An approach to overcome the drawbacks of ALA was the introduction of more lipophilic derivatives, eg, the MAL photosensitizing precursor. MAL was approved for PDT, not only for superficial lesions but also for the treatment of small nodular carcinomas due to its superior skin penetration compared with ALA.^{16,19} However, no significant difference in efficacy for treatment of actinic keratosis was demonstrated between ALA + PDT and MAL + PDT in a comparative study.³² MAL (Metvix® Photocure ASA, Oslo, Norway) is currently approved and commercialized in many countries (eg, US, Europe, and Brazil) whereas ALA is commercialized only in the US (Levulan®, Kerastic Dusa Pharmaceuticals Inc, Wilmington, MA).

The recent worldwide trend of nanoencapsulation of drugs has raised the question of whether a nanophotosensitizing precursor would be more efficient than a free photosensitizing precursor for evaluation, prevention, and treatment for field cancerization of actinic keratoses. In order to explore the outcomes of nanoencapsulation of photosensitizing precursors in more depth, ALA was selected for encapsulation in a nanoemulsion, whereas MAL was used as purchased on the market. At this point, it is important to emphasize that the literature has reported many comparative studies involving both free ALA and free MAL. Several advantages of MAL over ALA have been reported, including a higher permeation profile while crossing the stratum corneum and cell membranes, higher selectivity (eg, in solar actinic keratoses), increased formation of protoporphyrin IX leading to higher fluorescence and photodynamic efficiency, less pain, fewer systemic effects after local treatment, and faster clearance from cells and tissues.³³ For instance, a noduloulcerative basal cell carcinoma could not be sufficiently penetrated by ALA.²⁶ It was demonstrated that the fluorescence emitted from a skin

lesion when using ALA was less specific and less intense than when using MAL. Further, the occlusion time for ALA varied from 4 to 8 hours, whereas just 3 hours were needed for MAL. On the other hand, it was shown that ALA was more effective than MAL for the treatment of inflammatory lesions.¹⁹ However, *in vitro* experiments required a higher concentration of MAL than ALA for efficient formation of protoporphyrin IX.^{33,34} Indeed, the superiority of MAL over ALA is still an open question and many aspects need to be evaluated.³⁵

The aim of the present study was to carry out a pilot trial which included development of an efficient, robust, and inexpensive protocol for quantifying the time evolution of field cancerization for actinic keratosis using a combination of PDT, within a material platform of nanoencapsulation of a commercial photosensitizing agent, plus a new-developed software for photoimaging evaluation of as-treated field cancerization areas, by color image segmentation³⁶ using a new image filter and software especially developed for this purpose. The present study of field cancerization in actinic keratosis reports the outcome of PDT using the nanoencapsulated photosensitizer (nano-ALA) compared with MAL in promoting changes in the half-life of reduction in field cancerization, extent of the targeted area of field cancerization, and effectiveness for fractional reduction in the area of field cancerization. This study also demonstrates the robustness of the as-developed software for photoimaging evaluation of field cancerization, while providing a very effective and noninvasive tool for prevention and follow-up on a clinical basis.

Materials and methods

Two photosensitizers were used in the present study, ie, nano-ALA and MAL. Whereas commercial Metvix[®] incorporates 16.8% of MAL in its formulation, the as-developed nano-ALA was elaborated with 20% ALA. Nano-ALA encapsulates its photosensitizing agent onto a polymeric-based nanoemulsion produced from polylactide-polyglycol and egg-phosphatidylcholine lipids (50:50), with an average size ranging from 100 nm to 300 nm. The oil-in-water (o/w) polymeric nanoemulsion was prepared in the presence of ALA by a spontaneous emulsification process, as described in the literature.³⁷ In brief, the organic phase (acetone or mixture of organic solvents) containing medium-chain triglycerides, natural soy phospholipids, and ALA (Dye Pharmaceuticals, São José dos Campos, São Paulo, Brazil, a final concentration of 20%) was prepared at 55°C. Subsequently, this organic solution was added into the aqueous phase containing the anionic surfactant, poloxamer 188 (Sigma Aldrich, St Louis,

MO, USA), under magnetic stirring. The organic solvent was removed by evaporation under reduced pressure at 60°C for 6 hours. Formulations without the photosensitizing agent were prepared under the same conditions to be used as a reference for spectroscopic and photobiologic analyses.

This blinded-observer, intraindividual pilot study was carried out in three patients clinically diagnosed with bilateral field cancerization associated to actinic keratosis and/or photodamage on the face. Pre-existing clinically diagnosed lesions suspicious for basal cell carcinoma, Bowen's disease, or invasive squamous cell carcinoma were biopsied and treated appropriately within the protocols of the Skin Cancer Service at the Public Hospital, Brasília, Brazil. Patients with hypersensitivity to ALA or porphyrins, those undergoing immunosuppressive therapy, those suffering from porphyria, and those receiving systemically acting drugs with phototoxicity or photoallergic potential were excluded from participation in the study. To minimize the effects of chronic sun exposure that vary by side of the body depending on individual habits, eg, being a car driver or a passenger, and its possible consequences for determining the relative efficacy of nano-ALA and MAL, the three patients participating in this study were confirmed to have no particular sun exposure habits.

The patients selected for the study were booked for PDT and underwent a sequence of four PDT sessions, scheduled 3–4 weeks apart. Treatment options, risks, and the likelihood of success were fully explained to the patients, who agreed to undergo the PDT protocol, and read, discussed, and signed the consent form, which is part of the document file approved by the hospital's human ethics committee.

Prior to PDT, the patients' faces were cleaned with alcohol. A thin layer of the photosensitizing precursor (nano-ALA and MAL) was accurately applied on each patient's hemiface. The treated areas on both sides of the face were occluded using a plastic film as the first layer, followed by aluminum thin foil as the top layer. To treat the field cancerization, the appropriate occlusion time was established as being 2 hours.³⁸ After the occlusion period, the treated areas were uncovered and cleaned with gauze and physiologic serum. A Wood's lamp (model LY - 6MWD, Yoshi, São Paulo, Brazil) was then used to illuminate both sides of the treated faces while images of the fluorescent areas were digitally recorded using a Lumix-Panasonic[®] camera (model DMC-ZS1-12x, Osaka, Japan) in high exposure mode (flash off). The pictures were acquired at a fixed distance of 10 cm from the patient's face. In order to achieve the highest fluorescence image quality while recording the images, all light sources in the room except the Wood's lamp were turned off or covered with dark

material. In our analysis, a slight red (soft pink) fluorescence was established as the minimal fluorescence signal. The fluorescence was evaluated inside a chosen polygon connecting the angle of the mandible, the inner and outer corners of the eye, and the lip commissure.

Following this image recording step, the treated areas were illuminated for 10 minutes with a light-emitting diode (LED) source (Multiwaves[®], Indústria Mecânica Fina, São Paulo, Brazil) tuned to a wavelength of 630 nm (Red-Spot) and set at 11 mW/cm² and 13.8 J. A low fluence and short exposure time (10 minutes) were chosen to minimize any effects of PDT, allowing multiple treatment sessions in order to observe the time evolution of the field cancerization data in more detail. Exposure to the LED light protocol was performed on each hemiface while protecting the other hemiface from light. During application of the LED, the skin was kept cool with cold air (Siberian[®], Indústria Mecânica Fina) and cold water spray. Four PDT sessions, including applications of nano-ALA and MAL, Wood's illumination for image recording, and LED light treatment, were done in the three patients with a 3–4-week interval between sessions, depending on the patient's schedule for visiting the hospital service. Prior to all PDT sessions, the patients were clinically evaluated for both onset of new actinic keratoses and/or photodamaged areas, and the time evolution of the treated areas.

Fluorescence-sensitive imaging software was developed in order to analyze the time evolution of the as-treated areas in a quantitative manner using the protocol herein described, whether related to actinic keratosis or photodamage. In brief, this software loads the digitally recorded fluorescent areas under Wood's lamp illumination with the skills of sampling and using a new filter and using a new filter for colorimetric image segmentation based on red, green and blue (RGB) color space. The software evaluates the luminescent area within a region of interest, defined as the closed white polygon, as described later on in this paper. As mentioned above, the as-drawn polygon is based on anatomical aspects of the patient's face, and is roughly symmetrical. We choose the RGB color space for image processing due to its hardware-oriented nature that matches the way humans perceive colors, being sensitive to red, green, and blue components of visible light.³⁹ In addition, we implemented a feature in the as-developed software which allows the user to map the edges of field cancerization simultaneously in two selected images, ie, the original and the processed one. This feature allowed us to compare the results obtained using color segmentation by software with that obtained by human operators using edge recognition patterns while analyzing the healthy and field cancerization

regions of the patient's face. This was the approach used for evaluation of the quality of the segmentation process. A more detailed description of this software can be found in a recent patent filing.⁴⁰ For each hemiface, the total calculated areas are plotted against the duration of treatment (ie, PDT sessions) and the observed time decay is compared. Emphasis is on interpretation of the observed differences in the time decay constants (half-life) of field cancerization, extent of the targeted area of field cancerization, and fractional reduction in the area of field cancerization in terms of photosensitizing nanoencapsulation versus non-nanoencapsulation.

Results and discussion

Figure 1A shows a typical transmission electron micrograph of the as-prepared nanoemulsion, highlighting the suspended nanocapsule, with the phospholipid-based shell pictured as a dark ring surrounding the light inner oily content. Figure 1B shows a diagram of the nanocapsule suspended within the nanoemulsion, revealing the phospholipid structure of the shell of the nanocapsule with the lipophilic tails of the molecules facing the oily interior and the polar heads facing outwards. Figure 1C shows the typical hydrodynamic size distribution of the 5-aminolevulinic acid-loaded nanoemulsion obtained by dynamic light scattering (210 nm), while Figure 1D shows the typical zeta potential distribution of the 5-aminolevulinic acid-loaded nanoemulsion obtained by electrophoretic measurement (–51.4 mV).

Data on Table 1 shows the size increment of the ALA-loaded nanocapsules (210.2 nm average diameter) compared with unloaded nanocapsules (180.0 nm average diameter), thus supporting the anchoring of ALA molecules onto the external surface of the nanocapsule. Further, the average 15 nm increment in the thickness of the nanocapsule shell supports the hypothesis of the onset of a well structured bond-based interfacial domain, which we believe confers high stability to the nanostructured end product. Furthermore, the average zeta potential data included in Table 1 shows a substantial reduction, from –40.3 to –51.4 mV, as a result of ALA nanoencapsulation, representing a significant change to the external surface of the nanocapsule, believed to be due to anchoring of ALA molecules.

Following the protocol described earlier, Figure 2 shows a typical digital image of a fluorescent area on a patient's face while under Wood's lamp illumination. Although the skin itself has a fluorescence background, the protocol used in the present study allowed observation of both fluorescent and nonfluorescent areas, variations in these areas from patient to patient, and between MAL and nano-ALA photosensitizers.

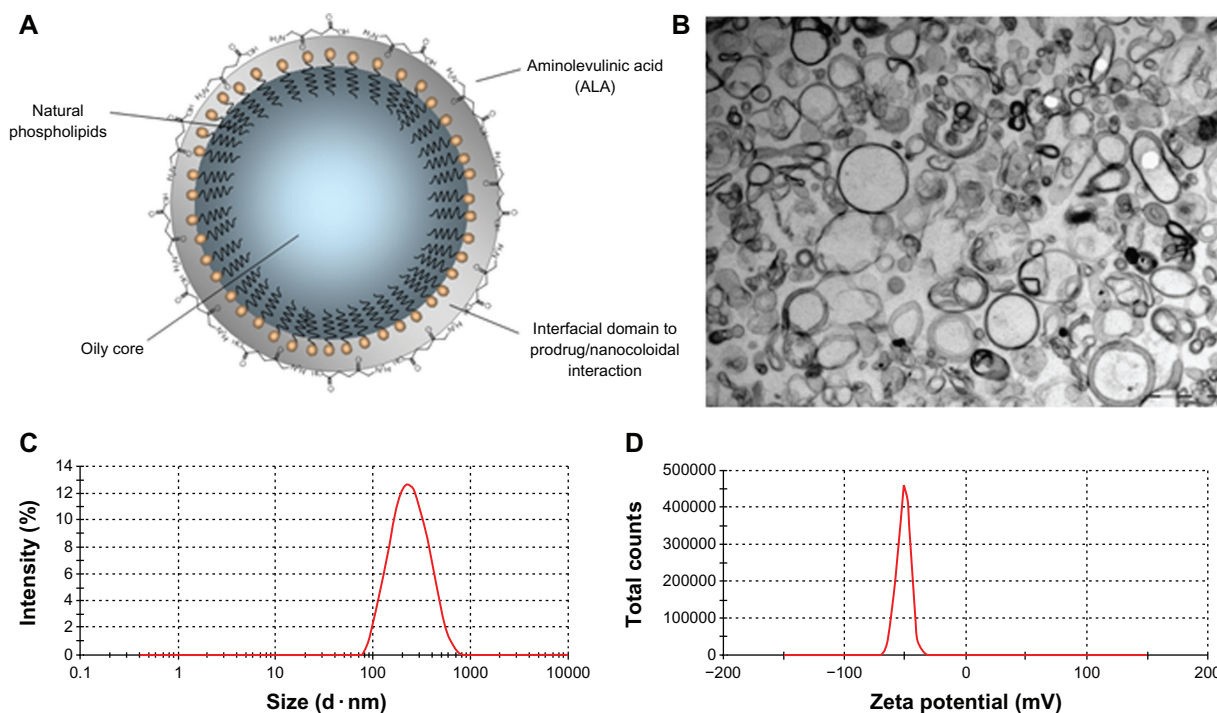


Figure 1 (A) Diagram representing the as-produced nanoemulsion, (B) typical transmission electron micrograph of the as-produced nanoemulsion, (C) typical hydrodynamic size distribution of the 5-aminolevulinic acid-loaded nanoemulsion obtained by dynamic light scattering, and (D) typical zeta potential distribution of the 5-aminolevulinic acid-loaded nanoemulsion obtained by electrophoretic measurement.

Notice the as-drawn polygon (solid white line) and the assessed inner luminescent area (red colored) in the left and right panels, respectively. Figure 3 shows the images of a typical time course (days) of treatment, as stated in the protocol for recording the pictures on both sides of the face under Wood's lamp illumination. At this point, we can report that the recorded luminescent area scales linearly with the extent of field cancerization, following the relationship observed between levels of protoporphyrin IX and clearance of lesions after photodynamic-MAL therapy.⁴¹

In order to carry out a quantitative study of the as-described PDT protocol for both treatment and evaluation of field cancerization associated with actinic keratosis, the time dependence of the luminescent areas recorded from all patients was collected and is shown in Figure 4, which

Table I Physicochemical parameters of nanocapsules loaded and not loaded with 5-aminolevulinic acid

Sample	NC	NC-ALA
Size (nm)	180.0	210.2
Size dispersion	0.08	0.16
Zeta potential (mV)	-40.3	-51.4
Solubility	Aqueous	Aqueous
Physicochemical stability (days)	90	90
PDT pathway mechanism	-	Type II (1O_2)

Abbreviations: NC, nanocapsules not loaded with 5-aminolevulinic acid; NC-ALA, nanocapsules loaded with 5-aminolevulinic acid; PDT, photodynamic therapy.

represents the luminescent area normalized with respect to the recorded area. The recorded area is the area (inner) delimited by the solid white line polygon (see Figure 2). The normalization procedure takes into account the luminescent area of the very first PDT session for each patient and each hemiface. The number associated with the normalized area represents the number of pixels associated with the luminescent area over the number of pixels associated with the as-drawn polygon (solid white line). It should also be noted that one single exponential decay function was used for fitting the experimental data for both nano-ALA and MAL, as shown in Figure 4, regardless of the intraindividual aspects of the data analysis. Nevertheless, the field cancerization time decay constants for the two formulations were different, being about 62.5 and 49.5 days for nano-ALA and MAL, respectively. This translates into a field cancerization half-life, respectively, of about 43.3 and 34.3 days for nano-ALA and MAL. Although the difference in reduction of the half-life of the field cancerization area is about 26% longer for the nano-ALA formulation, the average field cancerization area targeted by nano-ALA is around 45% higher than the average area targeted by the MAL formulation. In addition, for the time window presented in Figure 4, the average reduction in field cancerization area using the nano-ALA formulation is around 10% greater

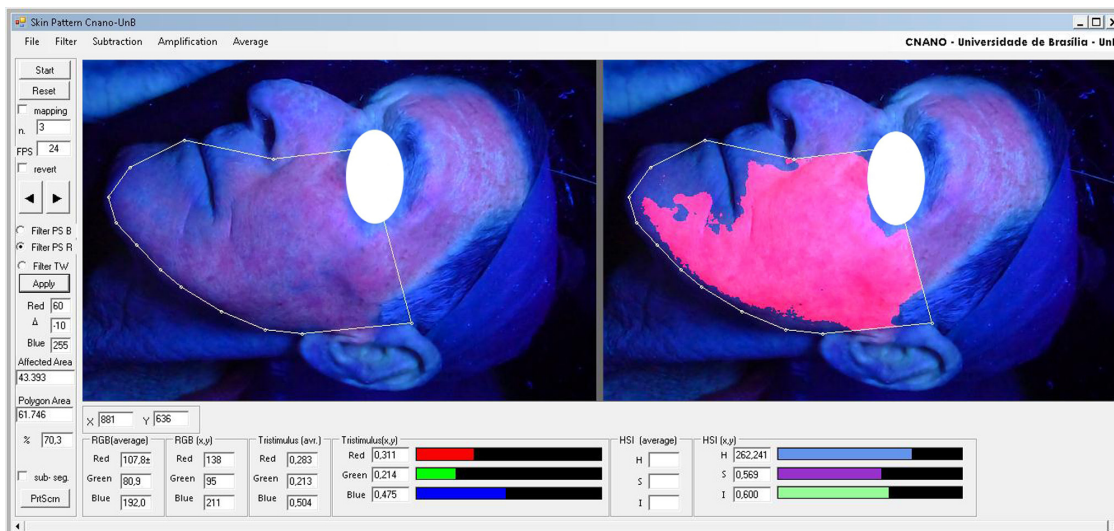


Figure 2 White polygon on both images defines the region of interest for color segmentation of field cancerization.

Notes: The left panel is the original image and the right panel is the treated image with the segmented area shown in red. Note that the software has a feature that allows the user to map the edges of field cancerization simultaneously in the two images in order to assess the quality of the segmentation process.

than the average reduction in field cancerization area using the MAL formulation. These findings have a wide range of fundamental and clinical implications.

From the clinical point of view, the largest field cancerization area targeted by the nano-ALA formulation (45%) as well as the slightly higher field cancerization area reduction (10%) represent an extremely valuable benefit in favor of ALA nano-encapsulation. However, the shortest reduction in half-life in field cancerization, apparently favoring the MAL formulation,

needs to be clarified by more indepth analysis, as indicated below. To have clinical application, the balance between the benefits of one approach versus the other should be weighed on a case-to-case basis by the attending physician. From the fundamental point of view, a relevant aspect of our findings is the difference between the two formulations (nano-ALA and MAL) in the extent of targeted field cancerization after occluding the treated area for only 2 hours, when the typical occlusion time reported in the literature for free ALA is more

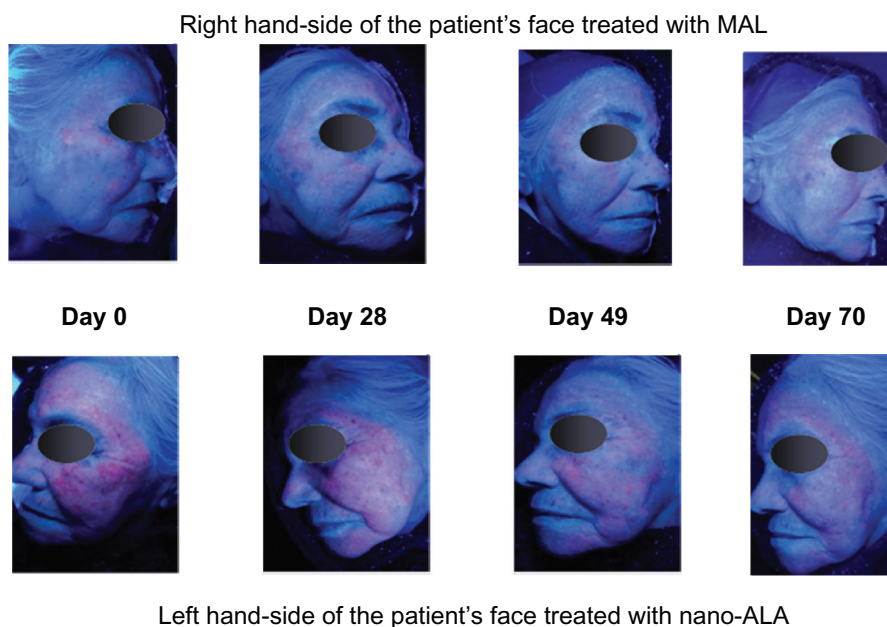


Figure 3 Sequence of images showing the time course of photodynamic therapy.

Note: The upper panel shows the right side of the patient's face (using MAL) and the lower panel shows the left side of the patient's face (treated with 5-aminolevulinic acid-loaded nanoemulsion).

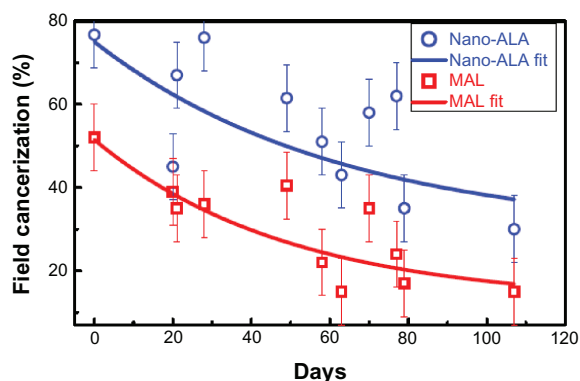


Figure 4 Time decay of field cancerization.

Notes: Symbols (open circles and squares) represent the experimental points and the solid lines going through the symbols represent the best curve-fitting according to time-dependent exponential decay. Symbols and the corresponding fitted solid lines are shown in blue for 5-aminolevulinic acid-loaded nanoemulsion (open circles) and red for MAL (open squares).

than 4 hours. We found from analysis of the data shown in Figure 4 that nano-ALA targets a larger area than MAL. This is indeed a key difference between the two formulations and has to do with the higher efficiency of the nano-ALA formulation in targeting field cancerization, indicating one benefit of nano-encapsulation. We argue that this targeting efficiency is more likely due to the greater diffusion of the nano-ALA formulation into the skin. Indeed, the literature reports high skin diffusion rates associated with drug-loaded nanocapsules and the key role played by the structure of the phospholipid-based shell.⁴² Based on this information, we believe that both the highest field cancerization area reduction (10%) and the longest reduction in half-life in field cancerization (about 26%) are consequences of the greater efficacy of the nano-ALA formulation in targeting field cancerization.

Conclusion

In summary, this paper introduces a novel, inexpensive, and simple approach that allows quantification of field cancerization associated with actinic keratosis and skin photodamage. Although current techniques allow monitoring of protoporphyrin IX fluorescence after application of a prodrug,⁴¹ no quantitative approach is available. Thus, the present proposed strategy fills an important gap in the literature as well as in the clinic. The protocol introduced herein is based on the quantitative evaluation of the fluorescent area after topical application of a photosensitizing precursor, following occlusion by 2 hours before using Wood's lamp illumination. The fluorescent skin area was digitally collected and its extent quantitatively evaluated using color image segmentation software especially developed for this application. Further, the approach introduced in this paper was used to assess information about the efficacy of skin field cancerization treatment using PDT.

Two photosensitizing precursors, namely the commercial formulation of MAL (Metvix) and a new nanostructured formulation for 5-aminolevulinic acid (nano-ALA), were used and comparatively evaluated in the pilot trial reported here. Our approach to evaluation of quantitative skin field cancerization allowed us to assess the information regarding the time decay constants (half-life) associated with reduction of the recorded luminescent area for both MAL (half-life 34.3 days) and nano-ALA (half-life 43.3 days). Further, our quantitative approach for probing skin field cancerization indicates that nano-ALA probes a 45% larger field cancerization area than MAL while using only 2 hours of occlusion. Finally, we found the reduction in area of skin field cancerization was 10% higher while using nano-ALA instead of MAL. Indeed, these findings have important implications, from fundamental aspects, eg, the changes in clinical response introduced by nanoencapsulation of a free drug, through to clinical aspects, eg, the feasibility of applying the approach presented here as a routine tool for detection, treatment, and follow-up of actinic keratosis and skin photodamage.

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Disclosure

A patent relating to this work has been filed, ie, de Souza PEN, Morais PC software for colorimetric segmentation, ColorSeg Instituto Nacional de Propriedade Industrial, register number 12876-4, February 24, 2012. Otherwise the authors report no conflicts of interest in this work.

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