



5-Aminolevulinic acid photodynamic therapy with and without Er:YAG laser for actinic keratosis: Changes in immune infiltration

Emese Gellén^{a,*}, Eszter Fidrus^a, Eszter Janka^a, Sándor Kollár^b, György Paragh^c, Gabriella Emri^a, Éva Remenyik^a

^a Department of Dermatology, Faculty of Medicine University of Debrecen, Hungary

^b Department of Pathology, Kenézy Gyula County Hospital, Debrecen, Hungary

^c Department of Dermatology, Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA

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ABSTRACT

Introduction: Ultraviolet light induced DNA damage, combined with immunosuppression and inflammation are involved in the pathogenesis of actinic keratosis. Photodynamic therapy not only destroys dysplastic cells via tissue destruction and vascular shutdown, but also induces an acute local inflammatory response and activates both the innate and adaptive immune system. In our current work we aimed to compare immunohistochemistry features of inflammatory infiltrate of actinic keratoses after 5-aminolevulinic acid photodynamic therapy with or without Er:YAG laser resurfacing.

Methods: Eleven patients with multiple actinic keratosis on the scalp, face, hands or forearms were treated by conventional and Er:YAG laser assisted 5-aminolevulinic acid PDT in split-site manner. Biopsies of AKs were taken before, 48 h and 3 months after the treatment. CD3, CD4, CD8, CD1a, Ki67 and p53 expressions were analyzed by immunohistochemical methods.

Results: The number of p53 and Ki67 positive cells decreased significantly 3 months after treatment, but the abnormal cells were not eliminated totally. The number of CD1a⁺ Langerhans cells significantly decreased 48 h after both treatments, while CD8⁺ T cell count was significantly lower 3 months after Er:YAG laser assisted photodynamic therapy. However, the number of CD3⁺ and CD4⁺ T cells were not changed significantly 48 h and 3 months later.

Conclusions: One session of 5-aminolevulinic acid photodynamic therapy even with Er:YAG laser pretreatment could not terminate actinic damage totally. Photodynamic therapy induced immunological changes. However further investigations are needed to answer how the composition of actinic keratosis' immune infiltrate influence the effect of photodynamic therapy.

1. Introduction

The presence of actinic keratoses (AKs) is a clinical sign of photocarcinogenesis. These precancerous lesions can lead to squamous cell cancer (SCC) [1]. A frequent first step in the pathogenesis of AKs is the ultraviolet light (UV) induced mutation of p53, which is followed by other gene mutations and epigenetic alterations, increased proliferation and decreased elimination of damaged keratinocytes, leading to AKs or even SCC [2]. Furthermore, UV-induced inflammation (COX-2) and immunosuppression (increased number of Foxp3⁺ T reg cells, isomerization of trans-urocanic acid to cis-urocanic acid) also contributes to photocarcinogenesis [2,3]. Multiple presentation within one area assigns field cancerization. In the concept of field cancerization, not

only the visible lesions, but the surrounding skin contains alterations as well, due to UV and environmental damage [3–5]. These mutated cell clones may initiate the development of new lesions and promote skin aging as well [3,6]. Therefore, to effectively eliminate AKs and to prevent the appearance of new ones, the whole area should be treated. It is also called field treatment [7–9].

Photodynamic therapy (PDT) is a field treatment modality. PDT shows very good therapeutic efficacy and excellent cosmetic outcomes [10,11]. Although, higher complete clearance rates are reported if PDT follows Er:YAG (erbium:yttrium-aluminium-garnet) or CO₂ ablative fractional laser (AFL) pretreatment [12,13]. The reason might be the higher penetration of photosensitizer through the ablative laser induced microscopic holes [12,13]. One major disadvantage of PDT is pain,

* Corresponding author.

E-mail addresses: emesegellen@gmail.com, emesegellen@med.unideb.hu (E. Gellén).

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however lately numerous techniques have been shown to markedly limit PDT related pain, including daylight PDT and two-step irradiance [14–16]. PDT induces intracellular reactive oxygen species and besides the induction of apoptosis and necrosis in dysplastic cells it also induces vascular shutdown, inflammatory and immunological changes [17–20]. Furthermore, PDT reverses photoaging through direct epidermal and indirect dermal changes. PDT induces fibroblast activation, neocollagenesis and collagen remodeling while pigmentary changes and epidermal architecture improve as well [21,22]. Interestingly, besides anticancer immunity PDT has also been shown to trigger local immunosuppression [23]. Although, PDT induces ROS it does not appear to be mutagenic and even lacks the ability to induce cyclobutan pyrimidine dimers in the presence of melanin [24].

PDT-induced in vitro and in vivo cell death has been widely investigated [25–27]. However, limited data is available on the immunological changes after PDT in AKs and skin cancer models [27], and the differences between the inflammatory infiltrate after conventional PDT and after PDT with fractional laser pretreatment are not known.

Therefore, the main objective of the present study was to analyze and compare the composition of immune cells infiltrating AKs before and after conventional and Er:YAG -AFL - assisted PDT in order to assess, how PDT with these modalities could change the quality and quantity of induced immune response. We also aimed to compare therapeutic efficacy and photorejuvenation effect of Er:YAG - AFL compared to conventional PDT (cPDT) in patients with multiple actinic keratosis during a 12-month follow up period.

2. Methods

2.1. Study population

Adult (> 18 years) patients at Department of Dermatology of the University of Debrecen with severely sun damaged skin and at least fifteen AKs on both forearms, dorsal hands, face or scalp were offered to participate in the study. The trial was approved by the Regional Ethical Committee (certificate number: 030174/2014/OTIG). The study was conducted in accordance with the Declaration of Helsinki. Patients gave their written informed consent. Eleven patients were included in the study.

Exclusion criteria included fever, pregnancy, lactation, history of porphyria or other photosensitive disorder (e.g. systemic lupus erythematosus), herpes simplex infection on the treated site, use of medications causing photosensitivity (e.g. retinoids, tetracycline, fluoroquinolones etc.), hypersensitivity to the photosensitizer (5-aminolevulinic acid), prior field treatment, uncontrolled neurological-, liver-, heart-, lung disorders, and chemotherapy or immunotherapy in the last 3 months.

2.2. Treatment protocol

Patient demographic data were recorded, photos were taken and AKs were counted and graded according to Olsen et al. [28] at baseline, 3 month and 12-month control. (Table 1) Treatment area were randomized to receive conventional PDT or Er:YAG-AFL PDT in random manner. One side was pretreated with Er:YAG-AFL (Sciton, ProFractional module, wavelength: 2940 nm; ablation depth: 30–100 µm; density: 22%) immediately before ALA application for laser assisted PDT, while the other side was subjected to conventional ALA PDT. For PDT, 20% of 5-aminolevulinic acid (5-ALA) (5-aminolevulinic acid hydrochloride Biochemica, AppliChem GmbH) was applied under occlusion on both side of the scalp, or face, or dorsum of the forearms and hands after curettage. The area then was irradiated with water-filtered infrared A light (Hydrosun® 501 halogen lamp with 4 mm water cuvette at 250 mW/cm² total irradiance intensity, waterfiltered spectrum 590–1400 nm) for 20 min after 3-h incubation time. All patients received one session of treatment without topical anesthesia and wore

protective goggles during illumination.

2.3. Clinical evaluation

Patients were evaluated 1, 3, 6, 9 and 12 months after treatment by the same investigator. The number and grade of AKs were determined on all visits on both treated sites without knowing the form of treatment. The objective signs of photoaging were evaluated before, 3 and 12 months after therapy. Five-point scale for photodamage - adapted from Dover et al. [29] and Zane et al. [30] - were applied to determine the objective signs of photoaging.

Skin biopsy samples (6 mm punch biopsies) of an AK and photographically verified sites of previous AKs were obtained after topical anesthesia (lidocaine hydrochloride) before PDT, 48 h and 3 months after PDT.

2.4. Immunohistochemical staining

Skin samples were fixed with 10% formaldehyde, embedded in paraffin and 3 µm-thick sections were prepared. The sections were deparaffinized, the endogenous peroxidase activities were inactivated in 3% H₂O₂ for 15 min. Antigen retrieval was achieved by pressure-cooking of tissue samples in Tris-EDTA buffer (pH 9). Sections were blocked with 1% fetal bovine serum (Biosera, Nuaille, France) in Tris-buffered saline (TBS) for 1 h in room temperature. Sections were stained with antibodies as follows: CD3 (Biocare Medical, Pacheco, CA, USA; clone EP41 ;1:100), CD4 (Leica Biosystems, Wetzlar, Germany; clone 4B12; prediluted), CD1a (Abcam, Cambridge, UK; clone C1A/711; 1:600), CD8 (Cell Signaling, Danvers, MA, USA; clone C8/144B; 1:1200), p53 (Biocare Medical, Pacheco, CA, USA; clone DO-7; 1:100), Ki67 (Biocare Medical, Pacheco, CA, USA; clone SP6; 1:100). Sections were incubated with the primary antibody overnight at 4 °C, after washing HRP-conjugated secondary antibody (One-Step Polymer HRP Reagent; BioGenex, CA, USA) was used for 30 min at room temperature. Staining was detected by DAB Chromogen (BioGenex, Fremont, CA, USA), Vector® VIP and ImmPACT™ NovaRED™ Kit (p53 and Ki67 staining, VECTOR Laboratories, Burlingame, CA, USA). Methyl green stain was also performed in p53 sections.

One of the serial sections prepared from the tissue blocks was stained with hematoxylin-eosin for histopathological evaluation.

The number of keratinocytes with positive p53 and Ki67 staining, the number of CD1a⁺ epidermal Langerhans cells, and dermal CD3⁺, CD4⁺ and CD8⁺ T cells were evaluated in blinded manner by a dermatologist and a pathologist. A score was calculated as the average number of cells in question per high power field (hpf, x100 magnification) counting the positively stained cells in three hpf for each section.

2.5. Statistics

The distribution of data was analyzed by Kolmogorov-Smirnov test. Fisher exact test was applied for the analysis of categorical variables. The therapeutic efficacy of the treatment modalities was assessed by the Wilcoxon matched-pairs signed rank test. Changes in cell numbers after the treatments was analyzed by Friedman test supplemented with Dunn's post hoc test.

We analyzed correlation between any therapeutic efficacy determined clinically, p53 and Ki67 immunopositivity and initial CD3, CD4, CD8 and CD1a cell counts using Spearman's rank correlation tests. Significance level was $p < 0.05$. Statistical analysis was performed by SPSS 25. (SPSS package for Windows, Release 25.; SPSS, Chicago, IL, USA).

Table 1
Patients characteristics.

Patient number	Age (years)	Gender	Treated area	Er:YAG – AFL-PDT									cPDT									
				Initial AK count and grade			AK count and grade after 3 months			AK count and grade after 12 months			Initial AK count and grade			AK count and grade after 3 months			AK count and grade after 12 months			
				GrI	GrII	GrIII	GrI	GrII	GrIII	GrI	GrII	GrIII	GrI	GrII	GrIII	GrI	GrII	GrIII	GrI	GrII	GrIII	
1	78	Female	Forearm	0	12	0	1	0	0	0	0	0	0	0	8	0	4	0	0	0	0	0
2	85	Male	Face	0	6	4	2	0	2	0	3	2	0	6	2	2	0	1	0	4	2	
3	82	Male	Scalp	10	8	1	0	1	0	3	0	0	8	9	0	3	1	0	3	0	2	
4	81	Male	Scalp	5	3	3	0	0	0	0	2	0	6	3	1	0	0	0	0	3	0	
5	72	Male	Hand	13	8	0	2	0	0	10	0	0	13	10	0	2	0	0	12	2	0	
6	81	Female	Forearm	22	6	0	0	0	0	12	2	0	20	7	0	1	0	0	9	0	0	
7	70	Female	Hand	6	5	0	0	0	0	0	2	0	4	5	0	1	0	0	0	4	0	
8	73	Female	Face	10	4	0	0	0	0	0	0	0	22	0	0	0	0	0	0	0	0	
9	87	Female	Face	23	3	2	7	6	0	24	4	0	22	4	0	10	3	0	19	1	0	
10	78	Female	Forearm	19	0	0	0	0	0	0	0	0	10	3	0	3	0	0	0	0	0	
11	64	Female	Hand	36	7	0	8	0	0	15	0	0	32	7	0	12	0	0	15	0	0	

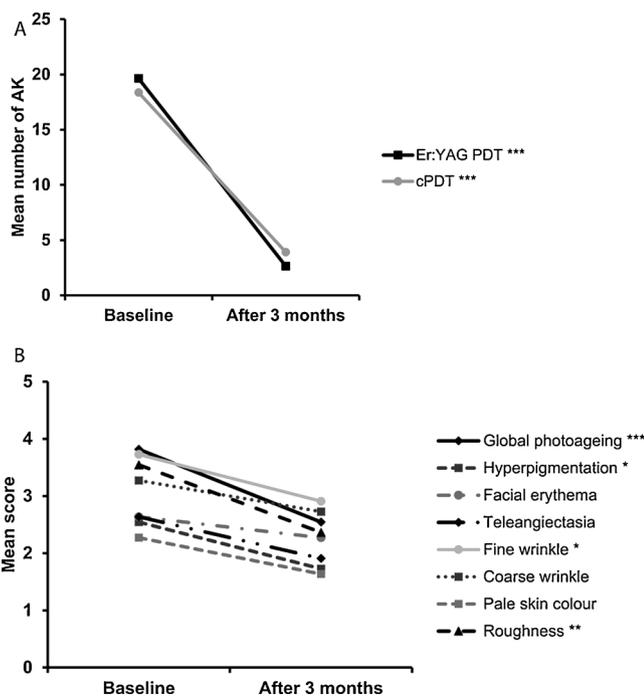


Fig. 1. a) Mean number of AKs before and after Er:YAG-AFL-PDT and cPDT *** p < 0.001. b) Photoaging * p < 0.05; **p < 0.01; *** p < 0.001.

3. Results

3.1. Study population

Eleven patients (average age 77 ± 6,9 years) with a total number of

427 AKs completed the study. The patients' characteristics are summarized in Table 1.

There was no statistically significant difference in the initial number of AKs on the two differently treated sides (p = 0.769).

3.2. Clinical efficacy

The number of AKs has significantly decreased 3 months after both Er:YAG-AFL PDT and cPDT by 87.56 ± 17.30% and 82.56 ± 16.53% (p = 0.039) (Fig. 1a). At 12 months follow up Er:YAG-AFL PDT and cPDT showed 69.45 ± 30.94% and 66.9 ± 25.41% (p = 0.844) decrease in the number of AKs, respectively.

Interestingly, Er:YAG AFL pretreatment induced higher therapeutic efficacy in those patients where PDT was highly effective (rs = 0.838, p = 0.002).

Both treatment modalities significantly improved global photoaging, mottled pigmentation, the roughness of the skin and both decreased significantly fine wrinkles 3 months later (Fig. 1b).

3.3. Histological evaluation

Analyzing hematoxylin-eosin stained tissue sections we found prominent dysplasia and solar elastosis, and a moderate inflammatory cell infiltration in AK samples before treatment. At 48 h after Er:YAG-AFL PDT or cPDT, a prominent inflammation with acantholysis and necrosis could be observed. 3 months later, both dysplasia and solar elastosis decreased (Fig. 2).

3.4. Immunohistochemical evaluation

Immune cell composition of an AK is represented on Fig. 3.

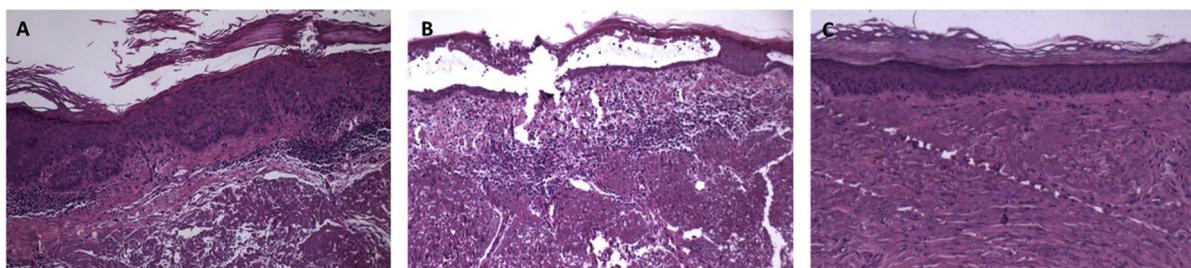


Fig. 2. Short- and long-term effect of Er:YAG-AFL-PDT on AK (H&E staining, 100x magnification). A: AK; B: 48 h after Er:YAG-AFL-PDT; C: 3 months after Er:YAG-AFL-PDT.

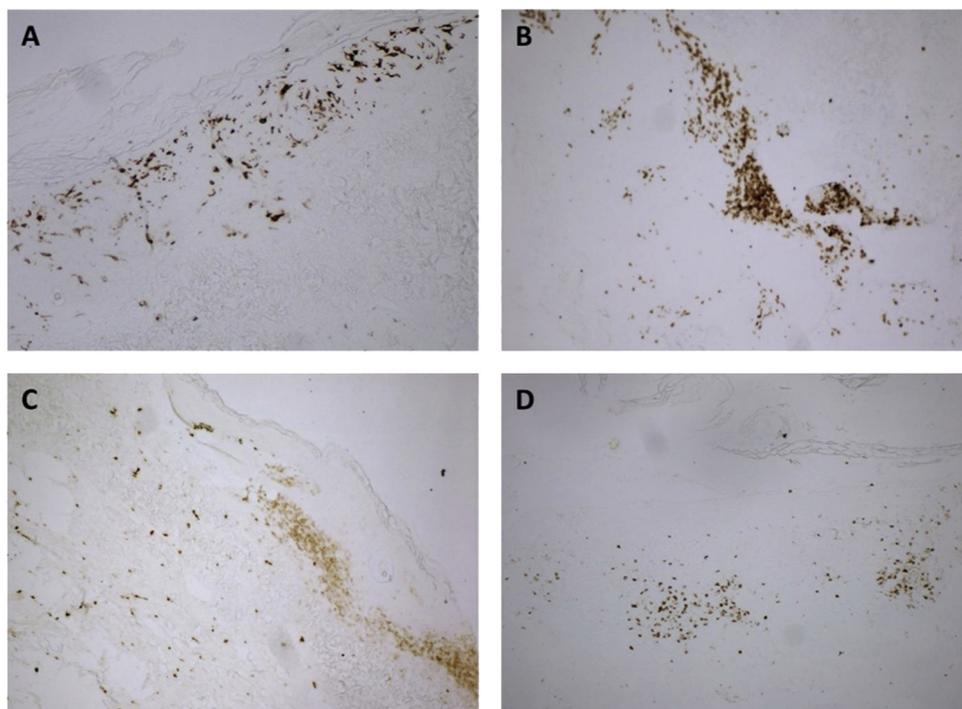


Fig. 3. Immune cell composition of AK (100x magnification, DAB chromogen). A: CD1a⁺ Langerhans cells; B: CD3⁺ T cells; C: CD4⁺ T cells; D: CD8⁺ T cells.

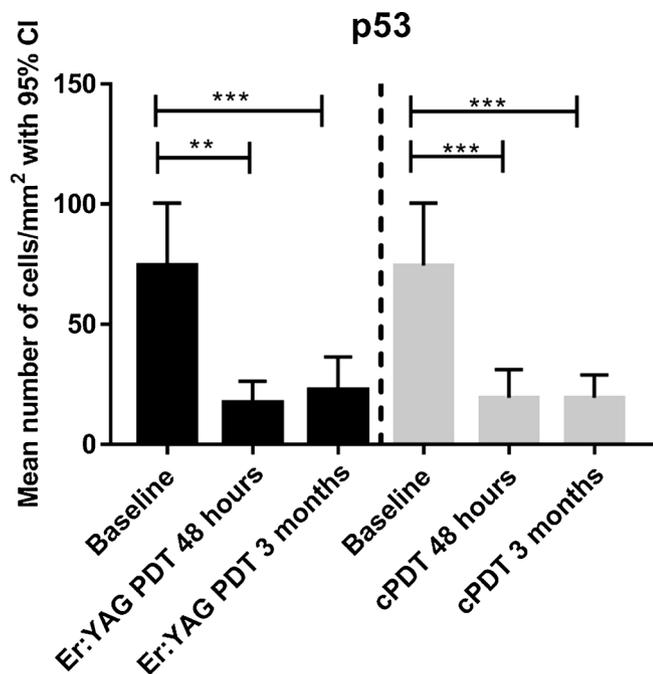


Fig. 4. Number of p53⁺ cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

3.4.1. Epidermal changes

3.4.1.1. p53 expression. The number of p53 positive keratinocytes significantly decreased at 48 h and it was also significantly lower at 3 months after both Er:YAG-AFL-PDT (p = 0.004 and p < 0.001) and cPDT (p < 0.001 and p < 0.001) compared to the corresponding initial AK field (Fig. 4). Significant difference was not seen between the two treatments at the time points (p = 0.559 and p = 0.651).

3.4.1.2. Ki67 expression. The number of Ki67 positive cells decreased

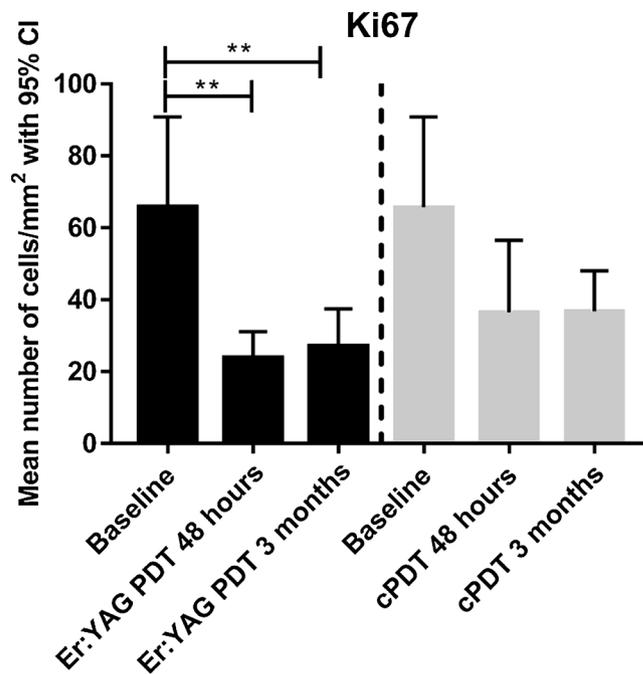


Fig. 5. Number of Ki67⁺ cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

significantly at 48 h (p = 0.002) and was significantly lower at 3 months (p = 0.009) after Er:YAG-AFL-PDT compared to the pre-PDT AKs (Fig. 5). The ratio of Ki67 immunopositivity also showed a trend towards decrease after cPDT but it did not reach statistical significance at either 48 h or 3 months (p = 0.099 and p = 0.057), respectively.

Fig. 6. demonstrates the changes in Ki67 and p53 expressions before and 3 months after the treatments.

3.4.1.3. CD1a⁺ Langerhans cells. The number of CD1a⁺ Langerhans

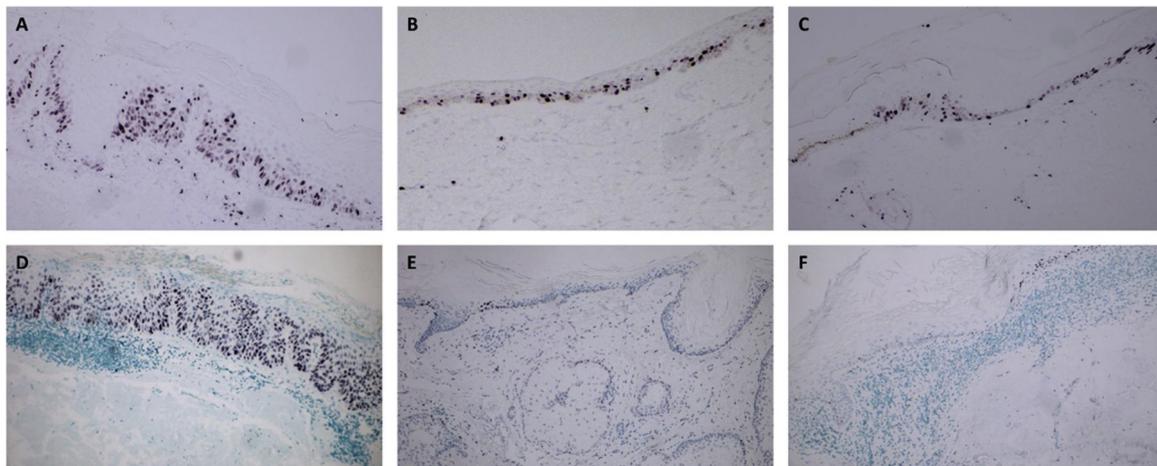


Fig. 6. Effect of Er:YAG-AFL-PDT and cPDT on the number of Ki67 and p53 positive cells (100x magnification, Vector VIP chromogen, methyl-green counterstaining). A: AK Ki67 expression; B: 3 months after Er:YAG-AFL-PDT Ki67 expression; C: 3 months after cPDT Ki67 expression; D: AK p53 expression; E: 3 months after Er:YAG-AFL-PDT p53 expression; F: 3 months after cPDT p53 expression.

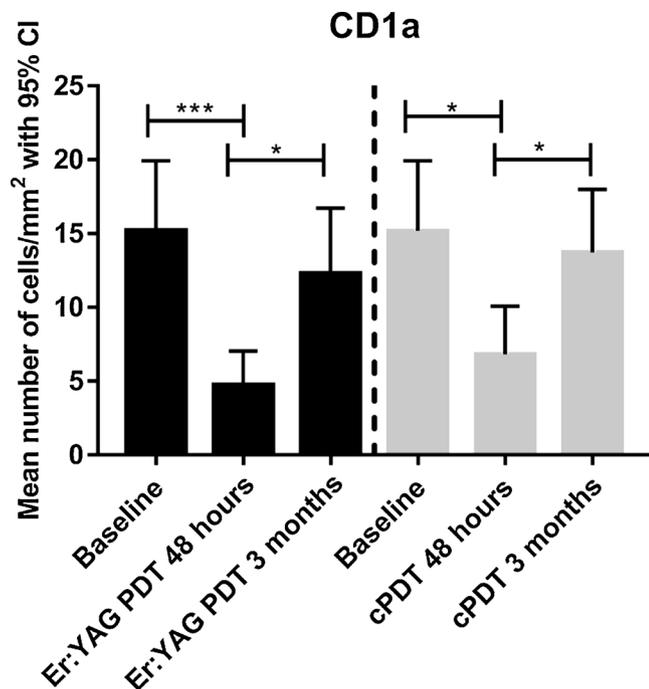


Fig. 7. Number of CD1a⁺ Langerhans cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

cells significantly decreased 48 h after both Er:YAG-AFL-PDT ($p < 0.001$) and cPDT ($p = 0.017$). At 3 months post-PDT their numbers returned almost to the initial level in both treatment groups (Fig. 7).

3.4.2. Dermal changes

We found positive correlation between the number of CD1a⁺ Langerhans cells and the number of CD3⁺ dermal T cells in all samples (baseline samples $r_s = 0.757$, $p = 0.009$, samples after 3 months Er:YAG-AFL PDT $r_s = 0.714$, $p = 0.016$; cPDT $r_s = 0.744$, $p = 0.011$). Interestingly, baseline CD3⁺ T cell count in AKs correlated with the decrease in p53 staining 3 months after Er:YAG-AFL PDT ($r_s = 0.683$, $p = 0.024$), and initial number of CD3⁺ T cells also correlated with 3 months therapeutic efficacy of cPDT ($r_s = 0.731$, $p = 0.013$).

Although there was no significant difference in the number of CD3⁺ and CD4⁺ T cells 48 h and 3 months after either Er:YAG-AFL- PDT

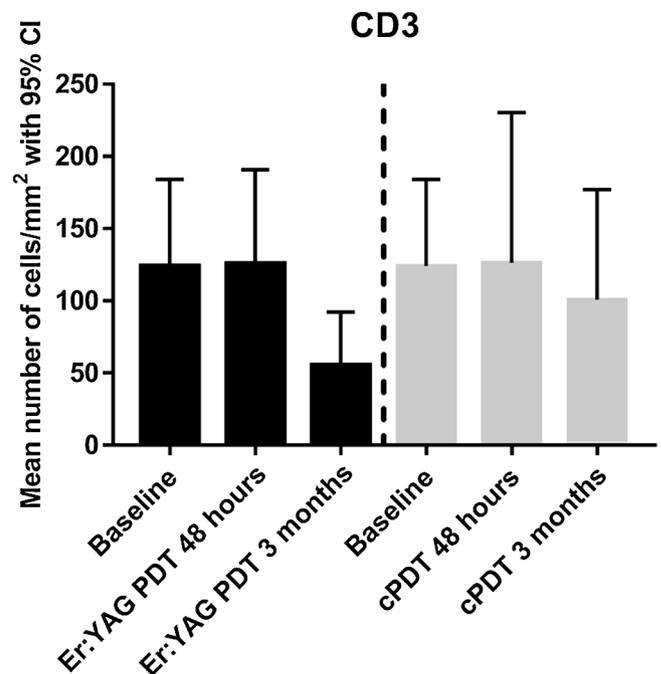


Fig. 8. Number of CD3⁺ T cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

($p = 0.998$ and $p = 0.103$; $p = 0.330$ and $p = 0.999$) or cPDT ($p = 0.999$ and $p = 0.872$; $p = 0.999$ and $p = 0.999$), we observed a decrease in the amount of CD3⁺ and CD4⁺ T cell infiltrate 48 h after both PDT treatments (Figs. 8 and 9). The number of CD8⁺ T cells also decreased 48 h after Er:YAG-AFL- PDT ($p = 0.274$) and cPDT ($p = 0.999$), and their number remained low 3 months later. While CD4⁺ T cell count reached almost the initial level at 3 months. In fact, the number of CD8⁺ T cells 3 months after Er:YAG-AFL- PDT was significantly ($p = 0.013$) lower than the baseline CD8⁺ T cell number (Fig. 10).

4. Discussion

The efficacy of PDT in the treatment of AK and field cancerization is well known, however recurrences occur and another session of field treatment is often needed [9–11,31]. Togsverd-Bo et al. reported first that ablative fractional laser pretreatment could enhance the clearance

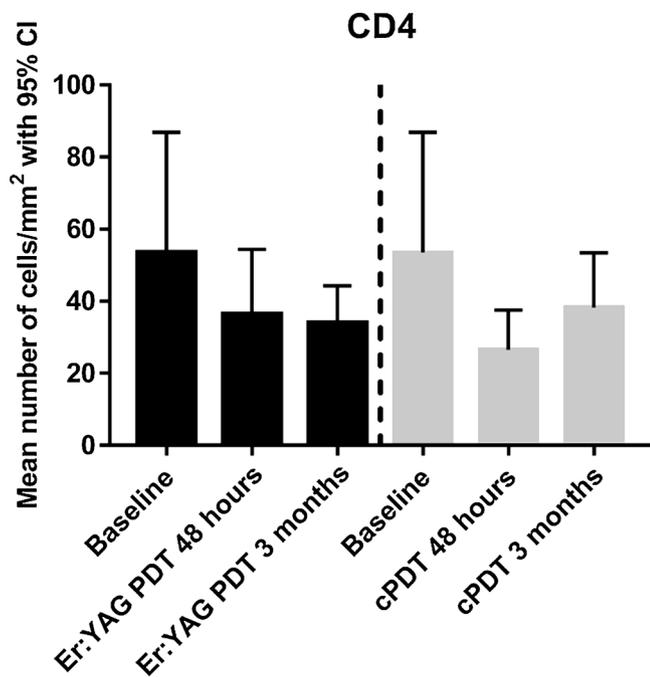


Fig. 9. Number of CD4⁺ T cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

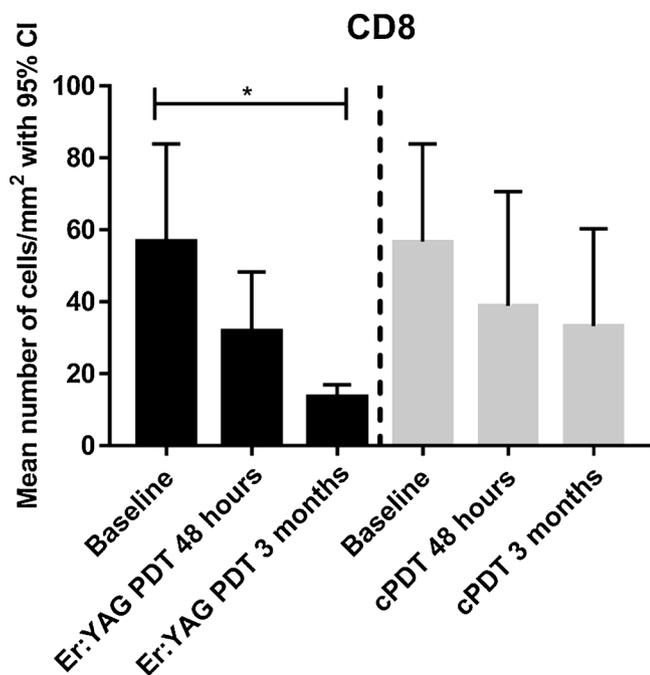


Fig. 10. Number of CD8⁺ T cells 48 h and 3 months after Er:YAG-AFL-PDT and cPDT.

of scalp and facial AKs and even prevent the occurrence of new lesions [32]. Higher complete clearance rates were seen by using both Er:YAG and CO₂ lasers before PDT [12,13,33,34]. Kim et al. reached 85.96% remission rate after 3 sessions of MAL-PDT with CO₂ laser pretreatment, while Ko et al. reported 86.9% complete response rate after one session of Er:YAG-AFL-PDT with 9.7% recurrence rate after 48 weeks [12,21]. Comparing the conventional 3 h incubation time with a shorter, 2 h incubation, Choi et al. found that 3h-Er:YAG-AFL-PDT had a higher efficacy (91.7%) than the 2h-Er:YAG-AFL-PDT (76.8%) [13]. In contrast, Vrani et al. applied fractional CO₂-laser assisted PDT after 1 h incubation time and 3 months later, 4 and 3 new AKs appeared at the

AFL-PDT and cPDT treated sites [34]. In our study we also found that fractional laser pretreatment lead to significantly higher clearance rates compared to cPDT. Moreover, the number of recurrent AKs was lower on the AFL-PDT sides.

In addition, both treatment modalities significantly improved global photoaging, mottled pigmentation, the roughness of the skin and both decreased significantly fine wrinkles 3 months later.

p53 protein is encoded by p53 tumor suppressive gene, and has a key role in cell cycle arrest, repair mechanisms and apoptosis. Its expression increases upon UV exposure. Nevertheless, the half-life of mutant p53 is longer than the wild type p53 protein, which can account for the high level of p53 expression in AKs and skin cancers. Ki67 is a marker for cell proliferation and increased expression was found in AKs as well [2,4,6]. Moreover, it showed correlation with p53 expression [35]. Through direct epidermal and indirect dermal effects, PDT could decrease the extent of keratinocyte atypia and solar elastosis and induce collagen production [36,37]. It could be revealed with decreased expression of p53 and Ki67 in the epidermis, even 6 weeks after the treatment, which reflect to an improvement in actinic damage [37,37]. With orcein and picrosirius staining, increased amount of collagen and decreased elastic fibers could be visualized 6 months after 2 sessions of MAL-PDT [38]. By immunohistochemistry, increased procollagen-I, and MMP-1 expressions could be observed 3 months after 3 MAL-PDT sessions [36]. While, 2 sessions of 5-ALA-PDT could increase the expression of procollagen-I, -III and decrease the expression of MMP-1, -3 and -12 [39]. These histological changes appear clinically as an improvement of skin texture, decrease of pigmentation, coarse and fine wrinkles, as we also observed in our clinical evaluation after a single treatment session [29,30,38].

In our study the number of abnormal keratinocytes characterized by positive Ki67 and p53 staining decreased 48 h after PDT. The composition of immune cells also changed 48 h later, the number of CD1a⁺ Langerhans cells decreased, besides a decrease in the number of CD4⁺ and CD8⁺ T cells, although the average CD4/CD8 ratio remained stable. There was no significant difference in the changes of immune infiltrate induced by Er:YAG-AFL PDT and cPDT. Other studies also confirmed the decrease of CD1a⁺ Langerhans cells in the skin 24 h after ALA-PDT [40,41]. In contrast to our results, 4 h after PDT the number of CD4⁺ and CD8⁺ T cells have been found to be increased in the skin, although the experiments were performed on healthy human skin and the shorter post PDT time may explain the difference between this observation and our findings [27,41]. In an SCC mouse model, 24 h and 1 week after ALA-PDT the amount of CD4⁺ and CD8⁺ T cells were also significantly increased [40,42]. Human studies, evaluating PDT induced changes in the immune infiltration of AK, to date are missing. Our findings suggest that PDT may eliminate not only the aberrant keratinocytes, but the originally present AK-associated immune cells as well. AK-associated immune cells may also be sensitive to PDT induced cell damage. At the same time, PDT initiates an inflammatory immune response, which could lead to immune activation or immunosuppression, as previous studies suggested [20,23]. We found that 3 months after PDT, the inflammatory infiltrate changed. The number of CD1a⁺ Langerhans cells returned to almost baseline. The average CD4/CD8 ratio increased. The number of CD8⁺ T cells further decreased 3 months after the treatment, probably these cells are responsible for the immune function against dysplastic keratinocytes in AKs. However, we have not found an association between PDT efficacy and initial number of CD8⁺ T cells. Nevertheless, it seems that the increased number of CD1a⁺ Langerhans cells and CD3⁺ T cells in AKs is associated with higher PDT efficacy. We found that the number of p53 and Ki67 positive epidermal cells has substantially decreased by PDT and Er:YAG-AFL PDT compared to the baseline, but the treatment did not eliminate the abnormal cells totally, suggesting that one treatment session is not enough to eliminate actinic damage, and therapy should be repeated.

Therefore, it is a legitimate assumption that the composition of immune infiltrate of AK influence the effect of PDT, or indirectly the

composition of immune infiltrate refers to the immunogenicity of cells constituting AK. Built in this, the efficacy of PDT could be predicted in the future at some level. Although more examinations are needed to confirm this supposition.

Conflict of interest

The authors have no conflict of interest.

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References

- [1] D.S. Riegel, L.F.S. Gold, The importance of early diagnosis and treatment of actinic keratosis, *J. Am. Acad. Dermatol.* 68 (1 Suppl. 1) (2013) S20–S27.
- [2] V. Ratushny, M.D. Gober, R. Hick, et al., From keratinocyte to cancer: the pathogenesis and modelling of cutaneous squamous cell carcinoma, *J. Clin. Invest.* 122 (2012) 464–472.
- [3] J. Wei, L.F. Kok, S.N. Byrne, et al., Photodamage: all signs lead to actinic keratosis and early squamous cell carcinoma, *Curr. Probl. Dermatol.* 46 (2015) 14–19.
- [4] B.J. Braakhuis, M.P. Tabor, J.A. Kummer, et al., A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications, *Cancer Res.* 63 (2003) 1727–1730.
- [5] A. Vierkötter, J. Kruttman, Environmental influences on skin aging and ethnic - specific manifestations, *DermatoEndocrinology* 4 (2012) 227–231.
- [6] R. Bosch, N. Phillips, J.A. Suárez-Pérez, et al., Mechanisms of photoaging and cutaneous photocarcinogenesis, and photoprotective strategies with phytochemicals, *Antioxidants* 4 (2015) 248–268.
- [7] E. Stockfleth, The importance of treating the field in actinic keratosis, *J. Eur. Acad. Dermatol. Venereol. (Suppl. 2)* (2017) 8–11.
- [8] G. Goldenberg, M. Perl, Actinic keratosis: update on field therapy, *J. Clin. Aesthet. Dermatol.* 7 (2014) 28–31.
- [9] R.N. Werner, E. Stockfleth, S.M. Connolly, et al., Evidence- and consensus-based (S3) Guidelines for the Treatment of Actinic Keratosis – international League of Dermatological Societies in cooperation with the European Dermatology Forum – short version, *J. Eur. Acad. Dermatol. Venereol.* 29 (2015) 2069–2079.
- [10] C.A. Morton, R.M. Szeimies, A. Sidoroff, et al., European guidelines for topical photodynamic therapy part 1: treatment delivery and current indications - actinic keratoses, Bowen's disease, basal cell carcinoma, *J. Eur. Acad. Dermatol. Venereol.* 27 (2013) 536–544.
- [11] N. Jetter, N. Chandan, S. Wang, M. Tsoukas, Field cancerization for management of actinic keratosis: a narrative review, *Am. J. Clin. Dermatol.* 19 (2018) 543–557.
- [12] D.Y. Ko, S.Y. Jeon, K.H. Kim, et al., Fractional erbium: YAG laser-assisted photodynamic therapy for facial actinic keratoses: a randomized, comparative, prospective study, *J. Eur. Acad. Dermatol. Venereol.* 28 (2014) 1529–1539.
- [13] S.H. Choi, K.H. Kim, K.H. Song, Efficacy of ablative fractional laser-assisted photodynamic therapy with short-incubation time for the treatment of facial and scalp actinic keratosis: 12-month follow-up results of a randomized, prospective, comparative trial, *J. Eur. Acad. Dermatol. Venereol.* 29 (2015) 1598–1605.
- [14] D.M. Rubel, L. Spelman, D.F. Murrel, et al., Daylight photodynamic therapy with methyl aminolevulinate cream as a convenient, similarly effective, nearly painless alternative to conventional photodynamic therapy in actinic keratosis treatment: a randomized controlled trial, *Br. J. Dermatol.* 171 (2014) 1164–1171.
- [15] J.M. Ang, I.B. Riaz, M.U. Kamal, G. Paragh, N.C. Zeitouni, Photodynamic therapy and pain: a systematic review, *Photodiagnosis Photodyn. Ther.* 19 (2017) 308–344.
- [16] G. Paragh, N.C. Zeitouni, Two-step irradiance treatment can achieve excellent pain control during red light 5-aminolevulinic acid photodynamic therapy for actinic keratoses, *Photomed. Laser Surg.* 36 (2018) 174–176.
- [17] A.D. Garg, D. Nowis, J. Golab, et al., Photodynamic therapy: illuminating the road from cell death towards anti-tumour immunity, *Apoptosis* 15 (2010) 1050–1071.
- [18] P. Fonda-Pascual, O.M. Moreno-Arrones, A. Alegre-Sanchez, et al., In situ production of ROS in the skin by photodynamic therapy as a powerful tool in clinical dermatology, *Methods.* 15 (109) (2016) 190–202.
- [19] B. Krammer, Vascular effects of photodynamic therapy, *Anticancer Res.* 21 (2001) 4271–4277.
- [20] E. Reginato, P. Wolf, M.R. Hamblin, Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects, *World J. Immunol.* 4 (1) (2014) 1–11 27.
- [21] S.K. Kim, G.B. Koo, Y.S. Kim, et al., Epithelial-mesenchymal interaction during photodynamic therapy-induced photorejuvenation, *Arch. Dermatol. Res.* 308 (2016) 493–501.
- [22] E. Kohl, L.A.R. Torezan, M. é mtsai Landthaler, Aesthetic effects of photodynamic therapy, *J. Eur. Acad. Dermatol. Venereol.* 24 (11) (2010) 1261–1269.
- [23] Y.J. Matthews, D.L. Damian, Topical photodynamic therapy is immunosuppressive in humans, *Br. J. Dermatol.* 162 (2010) 637–641.
- [24] S. Mudambi, P. Pera, D. Washington, É Remenyik, E. Fidrus, G. Shafirstein, D. Bellnier, Gy Paragh, Photodynamic therapy does not induce cyclobutane pyrimidine dimers in the presence of melanin, *Photodiagnosis Photodyn. Ther.* 22 (2018) 241–244.
- [25] H. Wang, J. Li, T. Lv, et al., Therapeutic and immune effects of 5-aminolevulinic acid photodynamic therapy on UVB-induced squamous cell carcinomas in hairless mice, *Exp. Dermatol.* 22 (2013) 362–363.
- [26] H. Nakaseko, M. Kobayashi, Y. Akita, et al., Histological changes and involvement of apoptosis after photodynamic therapy for actinic keratoses, *Br. J. Dermatol.* 148 (2003) 122–127.
- [27] E. Gellén, E. Fidrus, M. Péter, et al., Immunological effects of photodynamic therapy in the treatment of actinic keratosis and squamous cell carcinoma, *Photodiagnosis Photodyn. Ther.* 24 (2018) 342–348.
- [28] E.A. Olsen, L. Abernethy, C. Kulp-Shorten, et al., A double-blind, vehicle-controlled study evaluating masoprocol cream in the treatment of actinic keratoses on the head and neck, *J. Am. Acad. Dermatol.* 5 (1991) 738–743.
- [29] J.S. Dover, A.C. Bathia, B. Stewart, K.A. Arndt, Topical 5-ALA combined with intense pulsed light in the treatment of photoaging, *Arch. Dermatol.* 141 (2005) 1247–1252.
- [30] C. Zane, R. Capereza, R. Sala, et al., Clinical and echographic analysis of PDT using MAL as sensitizer in the treatment of photodamaged facial skin, *Lasers Surg. Med.* 39 (2007) 203–209.
- [31] R.M. Szeimies, R.T. Matheson, S.A. Davis, et al., Topical methyl aminolevulinate photodynamic therapy using red light-emitting diode light for multiple actinic keratoses: a randomized study, *Dermatol. Surg.* 35 (2009) 586–592.
- [32] K. Togsverd-Bo, C.S. Haak, D. Thaysen-Petersen, et al., Intensified photodynamic therapy of actinic keratoses with fractional CO2 laser: a randomized clinical trial, *Br. J. Dermatol.* 166 (2012) 1262–1269.
- [33] B.K. Kim, N.R. Lee, S.Y. Park, et al., Efficacy of photodynamic therapy with laser pretreatment for actinic keratoses and photorejuvenation as evaluated by fluorescent imaging, *Photodermatol. Photoimmunol. Photomed.* 31 (2015) 36–43.
- [34] F. Vrani, E. Sotiriou, E. Lazaridou, et al., Short incubation fractional CO2 laser-assisted photodynamic therapy vs. conventional photodynamic therapy in field-cancerized skin: 12-month follow-up results of a randomized intraindividual comparison study, *J. Eur. Acad. Dermatol. Venereol.* (June (4)) (2018), <https://doi.org/10.1111/jdv.15109>.
- [35] P.M. Carpenter, K.G. Linden, C.E. McLaren, et al., Nuclear morphometry and molecular biomarkers of actinic keratosis, sun-damaged, and nonexposed skin, *Cancer Epidemiol. Biomarkers Prev.* 13 (2004) 1996–2002.
- [36] R.M. Szeimies, L. Torezan, A. Niwa, et al., Clinical, histopathological and immunohistochemical assessment of human skin field cancerization before and after photodynamic therapy, *Br. J. Dermatol.* 167 (2012) 150–159.
- [37] L. Bagazgoitia, J. Cuevas Santos, Á Juarranz, P. Jaén, Photodynamic therapy reduces the histologic features of actin damage and the expression of early oncogenic markers, *Br. J. Dermatol.* 165 (2011) 144–151.
- [38] M.C. Issa, J. Pineiro-Maceira, M.T. Vieira, et al., Photorejuvenation with topical methyl aminolevulinate and red light: a randomized, prospective, clinical, histopathologic, and morphometric study, *Dermatol. Surg.* 36 (2010) 39–48.
- [39] M.Y. Park, S. Sohn, E.S. Lee, Y.C. Kim, Photorejuvenation induced by 5-aminolevulinic acid photodynamic therapy in patients with actinic keratosis: a histologic analysis, *J. Am. Acad. Dermatol.* 62 (2010) 85–95.
- [40] H. Wang, J. Li, T. Lv, et al., Therapeutic and immune effects of 5-aminolevulinic acid photodynamic therapy on UVB-induced squamous cell carcinomas in hairless mice, *Exp. Dermatol.* 22 (2013) 362–363.
- [41] G. Evangelou, M.D. Farrar, R.D. White, N.B. Sorefan, K.P. Wright, K. McLean, S. Andrew, R.E.B. Watson, L.E. Rhodes, Topical aminolevulinic acid– photodynamic therapy produces an inflammatory infiltrate but reduces Langerhans cells in healthy human skin in vivo, *Br. J. Dermatol.* 165 (2011) 513–519.
- [42] A.K. Bhatta, P. Wang, U. Keyal, et al., Therapeutic effect of Imiquimod enhanced ALA-PDT on cutaneous squamous cell carcinoma, *Photodiagnosis Photodyn. Ther.* 23 (2018) 273–280.