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Research Article

Prevention Strategies in Patients affected by Actinic Keratosis of the Head: A 12-Month, Prospective, Assessor-Blinded, Controlled Study with Lesion-Directed Treatment Associated with Medicalized Photoprotection

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ABSTRACT

Objective: We assessed the efficacy of a 100+SPF sunscreen containing DNA-repairing enzymes (endonuclease, glycosylase, and photolyase) and a potent antioxidant extract (Polypodium Leucotomos Extract: PLE) ("Medicalized" Photoprotection: MP), in subjects with Actinic Keratosis (AK) treated with cryotherapy measuring the evolution of AKASI score and through a non-invasive analysis with Line-field Confocal-Optical Coherence Tomography (LC)-OCT.

Patients and Methods: A prospective, 12-month, assessor-blinded, controlled study enrolling patients with ≤ 5 actinic keratoses on the face and/or scalp suitable for cryotherapy was performed. After cryotherapy, 30 subjects were instructed to apply the MP twice daily for 12 consecutive months. Thirty subjects were selected as a control group. AKASI score was evaluated at baseline, after 3 and 12 months.

Results: MP was associated with a significant reduction ($p<0.05$) of AKASI score after 3 and 12 months. The score of the treatment group doesn't differ from the control group one at baseline, but it was significantly lower after 12 months. LC-OCT evaluation showed, at the end of treatment period in comparison with baseline that the thickness of the epithelium and the stratum corneum significantly decreased. A significant decrease of disarranged epithelial architecture, dyskeratotic keratinocytes, atypical nuclei and dilated vessels has been also observed. A significant increase ($p=0.045$) of the Outlined Dermo-Epidermal Junction (ODEJ), was observed.

Conclusion: PLE-based MP significantly improves AKASI score in subjects after cryotherapy treatment in comparison with controls. In addition, our study showed that morphological features identified by LC-OCT could be potentially useful for the follow-up of AK subjects.

Keywords: Actinic keratosis; Lesion-directed therapies; Cryotherapy; Photoprotection; Actinic Keratosis Area and Severity Index (AKASI)

INTRODUCTION

Actinic Keratoses (AKs) are skin lesions deriving from clonal DNA alterations of intraepidermal keratinocytes, determining the development of varying degrees of dysplasia [1]. First described by Dubreuilh, [2], in the XIX century, who considered it a pre-neoplastic lesion, it was Ackerman, et al. [3], a century later, which overturned this concept, asserting that "no distinct histopathologic boundary exists. The reason is that solar keratosis is a squamous cell carcinoma, albeit an embryonic one". In more detail, AK is intraepithelial proliferations of dysplastic keratinocytes with potential for malignant

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transformation [4]. The evolutive risk and the costs associated with treatment, require improving early diagnosis and primary prevention [5]. New emerging non-invasive skin imaging techniques provides either a diagnostic support or a better clinical-instrumental follow-up, especially in evaluating the efficacy of a treatment [6]. Primary prevention is relying upon education of the population on the right UV exposure; hence, photoprotection with sunscreens is strongly recommended in AK subjects, especially after lesion-directed (i.e., cryotherapy) or field-directed (i.e., Photodynamic Therapy, PDT) treatments [7,8]. Recently, high-protection sunscreen products containing DNA-repairing enzymes and/or antioxidant molecules have shown to be able to reduce AK lesions and their recurrences, after conventional PDT [9]. So far, no prospective controlled data regarding AK treatment with lesion-directed therapies associated with Medicalized Photoprotection (MP) are available. To quantitatively evaluate the severity of AK across an entire affected field, the "Actinic Keratosis Area Severity Index" (AKASI), has been proposed and commonly used [10]. The main risk factor is UV radiation; although, other individuals' aspects such as fair skin, age, gender, inflammation, and immunosuppression, are involved into etiopathogenesis [11,12]. AKs affect mostly chronically sun-damaged skin of Fitzpatrick phototype I and II. The lesions appear rarely isolated, usually coexisting with other visible and sub-clinical ones, giving rise to a condition described as a "field cancerization" [13]. The worldwide prevalence is 11%-25%, whereas in Europe, is up to 49% in men and 28% in woman, with an average age of 71 years, and they are among the most common conditions seen in daily dermatological practice [14,15]. Several clinical and histological classification systems have been formulated for grading AK, although their use in clinical practice remains controversial, because of the poor agreement [16,17]. The main limitation of these scoring systems is the focus on the severity of a single lesion, without considering the entire area affected. To overcome this constraint, a new evaluation method, the "Actinic Keratosis Area Severity Index" (AKASI), has recently been proposed [10]. It allows to quantify the severity of AK across an entire affected field and to compare diseases's severity before and after the therapy, resulting more useful in daily routine [18]. Although the progression of a single AK to Cutaneous Squamous Cell Carcinoma (cSCC) is unpredictable and highly variable, it seems to range from 0.025% to 16% per year, whereas a patient with multiple AKs may have an annual risk of developing iSCC ranging from 0.15% to 80% [19]. Considering the lack of predictive features of the development of cSCC, early diagnosis is critical and the treatment of all actinic keratoses is strongly recommended [20]. Noninvasive imaging techniques such as dermoscopy, reflectance confocal microscopy [21], and more recently, Line-field Confocal-Optical Coherence Tomography (LC-OCT) [22], can be used to help the clinical diagnosis. Nowadays, the malignant evolutive potential and the costs associated with the disease, created a substantial financial burden on our healthcare system [23]. The education on the right UV exposure management and the regular use of sunscreens, it is strongly suggested in Actinic Keratosis (AK) subjects, especially after specific lesion-directed (i.e., cryotherapy) or field-directed (i.e., Photodynamic Therapy, PDT) treatments [24,25]. High-protection sunscreen products containing DNA-repairing enzymes and/or antioxidant molecules, have recently been formulated and have shown to be able to reduce AK lesions and, in the medium-term, recurrence lesions, after conventional PDT [9]. A new medical device containing a complete DNA-repair enzyme complex including photolyases, glycosylases and endonucleases, a combination of filters with full spectrum sun protection (UVB and UVA, SPF 100+), and an extract of Polypodium leucotomos leaves (Polypodium Leucotomos Extract: PLE, Fernblock® [26,27]) has been recently developed (Heliocare 360° MD AK, Cantabria Labs, Madrid, Spain). PLE has well-known antioxidant, photoprotective, and immune-modulatory activities [28-30]. The study aim was to realize a prospective controlled trial, to assess the biological effects of 12-month treatment with this sunscreen, on the evolution of AKASI score in AK subjects treated with cryotherapy, and to evaluate "*in vivo*" and not invasively the morphological changes of the lesions treated, with a new technology named Line-field Confocal-Optical Coherence Tomography (LC-OCT) [31,32], identifying AK criteria, before and at the end of the treatment period, by two investigators expert in skin imaging.

MATERIALS AND METHODS

Patients and setting

A 12-months prospective controlled assessor-blinded trial was performed, covering the period from May 2020 to May 2021, at the Dermatologic Unit of the University Hospital of Siena in 60 AK subjects. The study was conducted in accordance with the Declaration of Helsinki, [33], and has recruited 30 patients of both sexes, without history of immunosuppression, aged >60 years, with ≤ 5 clinically diagnosed, visible and/or palpable AKs, on the face and/or scalp, suitable for cryotherapy. Thirty AK patients, matched for age and sex, who refused to treat their lesions because of the lack of discomfort related, have been selected as the control group. All the sixty patients involved in the study investigation gave their informed and signed consent.

Treatment

AKs were outlined with a marking pen; one of them was biopsied, if considered a suspicious lesion, whereas all the other AKs were treated in the same session. Patients underwent cryotherapy using Liquid Nitrogen (LN) as cryogen, directly sprayed onto the lesion through an appropriate sized nozzle, held 1 cm from the skin surface. LN was delivered at -196°C, and ideally it reached approximately -50°C in contact with the skin, determining epidermal keratinocytes death. A double freeze-thaw cycle applied for 20 seconds was used. It involves a 1 mm rim of normal skin outside the marked outline of the lesion. After treatment, all the subjects in the active group were instructed to apply twice daily (morning and evening) the medicalized photoprotection on the face and scalp (2 Fingertip Units to be applied for every application) for 12 consecutive months. All samples for photoprotection were given to the patients at baseline visit. In the control group simply photoprotection advice were given (seeking shadow, use hat in

outdoor activities etc). The freely use of "non-medicalized" sunscreens in the control group was also allowed.

Assessment

AKASI score was evaluated and calculated at baseline, month 3 and month 12 by an assessor unaware of the type of treatment. Score determination implied the subdivision of the head into four areas and to size of the affected area was attributed a percentage value that was then represented by a numerical value between 0 and 6; as well as the severity of specific clinical signs (distribution, erythema and thickness) was assessed and scaled (zero to four, i.e., none to severe). An AKASI subscore was calculated for each of the four areas of the head and multiplied the subtotal by the area coefficient. The subtotals together give a total AKASI score for the entire head. Total scores range from zero (no AK/no actinic damage) to 18 (AK of the severest possible degree).

Line-field confocal optical coherence tomography device

LC-OCT is a time-domain OCT technology that uses line illumination of the skin and line detection of the signal rather than point scanning/detection [34]. LC-OCT (DAMAE Medical, Paris, France) produces vertically orientated sectional images and videos of the skin in a gray scale. Images/videos were acquired by two investigators, expert in skin imaging (E.C and A.L). The device is composed of a handheld probe connected to a central unit and a display. The device has 1.1 µm axial resolution, 1.3 µm lateral resolution, 500 µm scanning depth, 1.2 mm lateral field of view and10 frames/second acquisition.

LC-OCT image acquisition protocol

A drop of paraffin oil was placed between the lesion and the glass window at the tip of the LC-OCT camera to ensure refractive index matching. Live images were directly visualized on the screen, while the operator gently moved the tip of the probe over the lesion. At least four LC-OCT images and two videos were saved for each lesion.

Image evaluation

Two observers blinded to any clinical data evaluated all lesions for the presence/absence of each LC-OCT AK criteria as recently reported by Cinotti, et al. [35], represented by epidermis thickness, stratum corneum thickness, parakeratosis, ulceration, acanthosis, disarranged epidermal architecture, dyskeratotic keratinocytes, visible nuclei, atypical nuclei, dilated linear vessels, elastosis and Outlined Dermo-Epidermal Junction (ODEJ).

Statistical analysis

Statistical analysis was performed using R statistical software. Continuous variables were expresses as mean ± Standard Deviation (SD). Previous descriptive analysis was carried out, absolute frequencies and their percentages were calculated for qualitative variables, mean and standard deviation for quantitative ones. ANOVA for the repeated measures was carried out to evaluate the AKASI increase/decrease in time. Two-way mixed ANOVA was performed to evaluate if the information available could modify the progression. Multiple paired and unpaired t-tests with Bonferroni correction were carried out as post hoc analysis to compare the AKASI between times or between groups. Paired t-test, Chi-square and McNemar tests were performed to evaluate the OCT features between baseline and 12 months. Each statistical test was two-tailed and was considered significant for p-values<0.05. The primary endpoint of the trial was the evolution of AKASI score from baseline and after 12 months in both active and control groups. We have hypothesized that the tested treatment could reduce the AKASI score of at least 1.5 points in comparison with baseline and control group. With an effect size (Cohen's d value) of 0.75, with an alpha value of 0.05 and a power of 90%, a total of at least 60 subjects should be enrolled to detect this difference. The sample size was calculated using G-Power statistical software version 3.9 (Kiel, Germany).

RESULTS

The treatment group was constituted of 30 (83.3% men) subjects aged 78.63 ± 6.6 years. Eighteen out of 30 had histology examinations; thirteen had AK, four SCC and one traumatic ulceration. Fifteen (50%) had a previous history of non-melanoma skin cancer. In the treatment group the Olsen score was evaluated, and 21 patients had Olsen score equal to 2 and 9 patients to 3 (mean ± SD 2.26 ± 0.52). The control group was composed of 30 men with a mean age of 76.5 ± 3.6 (t-test p-value=0.123) and are all AK. Table 1 show that the AKASI in the treatment group significantly decreases over the time. Already after 3 months it decreased significantly (4.73 ± 2.07 *vs.* 6.06 ± 2.34; p=0.001) and then remains constant over time. In the treatment group only two subjects had a recurrence with an AKASI increase of +14% and +23%, five subjects patients showed an AKASI score increase of less than 10%.The AKASI of the treatment group was not different from that of the control group at baseline, but it was significantly lower after 12 months (p<0.05). The AKASI decrease was not influenced by other parameters such as sex, previous history of non-melanoma skin cancer or Olsen. Table 2 shows the LC-OCT changes between baseline and 12 months in the treatment group. The thickness of epithelium and the stratum corneum thickness significantly (p=0.001) decreased. After 12 months we observed a percentage reduction of parakeratosis (p=0.07). We could also detect a significant decrease of epithelial architecture disarrangement, dyskeratotic keratinocytes, and atypical nuclei and dilated vessels. The ODEJ, instead, significantly increased (p=.045).

AKASI	Baseline	3 months	12 months	p-value
100+SPF group (N=30)	6.06 ± 2.34	4.73 ± 2.07	4.63 ± 2.42	<0.001[a,b]
Control group (N=30)	6.08 ± 1.06		6.15 ± 1.14*	*<0.05

Note: [a]: Baseline AKASI score was statistically different in comparison with 3-month AKASI score value. [b]: BaselineAKASI score was statistically different in comparison with 12-month AKASI score value. *: Therapy group and control group are statistically different (p<0.05).

Table 1: Average AKASI score mean ± SD values between time and groups.

	Baseline	12 Months	p-value
Thickness epithelium (max)	119, 6 ± 47.7	89, 1 ± 27.5	0.001
Thickness stratum corneum (max)	62, 2 ± 67.5	39, 6 ± 46.3	0.001
Parakeratosis			
0	10 (33.3%)	17 (56.7%)	0.07
1	20 (66.7%)	13 (43.3%)	
Ulceration			
0	28 (93.3%)	30 (100.0%)	0.09
1	2 (6.7%)	0 (0.0%)	
Acantosis			
0	6 (20.0%)	8 (26.7%)	0.5
1	24 (80.0%)	22 (73.3%)	
Disarranged epithelial architecture			
0	11 (36.7%)	21 (70.0%)	0.01
1	12 (40.0%)	3 (10.0%)	
2	7 (23.3%)	6 (20.0%)	
Diskeratotic keratinocytes			
0	21 (70.0%)	29 (96.7%)	0.005
1	9 (30.0%)	1 (3.3%)	
Visible nuclei			
0	2 (6.7%)	0 (0.0%)	0.15
1	28 (93.3%)	30 (100.0%)	
Atypical nuclei			
0	6 (20.0%)	14 (46.7%)	0.01
1	15 (50.0%)	5 (16.7%)	

2	9 (30.0%)	11 (36.7%)	
Dilated vessels			
0	11 (36.7%)	23 (76.7%)	0.01
1	19 (63.3%)	7 (23.3%)	
Elastosis			
0	10 (33.3%)	11 (36.7%)	0.5
1	20 (66.7%)	19 (63.3%)	
ODEJ (Outlined Dermo-Epidermal Junction)			
0	11 (36.7%)	4 (13.3%)	0.04
1	18 (60.0%)	26 (86.7%)	
2	1 (3.3%)	(0.0%)	

Note: For binary classification: 0=absent; 1: Present; for 3-point classification: 0: no, 1: Mild; 2: Severe.

Table 2: OCT parameters before and after treatment.

DISCUSSION

AK results from the adverse effects of UV radiation on keratinocytes DNA which acts as initiating factor of a multifactorial cascade of molecular, cellular, viral, immune, and genetic events that influence the progression of AK to cSCC [36]. There are no defined features to predict which AKs are at increased risk for malignant transformation and related to a major morbidity and mortality. Thus, prompt treatment of all AKs is essential to prevent the risk of evolution. Several guidelines concerning AKs treatment have been published in the past years and distinguished among of lesion and field-directed therapies [37,38]. There are currently no clear guidelines regarding the specific density of AK lesions per unit skin area that would indicate a move from lesion-directed to field-directed therapy, but it is usually a decision made on clinical judgment going beyond density and clinical manifestation of the lesion, to the evaluation of tolerability, cost of the treatment, age, immune system activity, and compliance of the patient. International guidelines [39,40] on the management of AKs recommend for single lesions, the cryotherapy (liquid nitrogen) treatment. Cryotherapy is a lesion-directed treatment consisting of a focal ablative procedure that eliminates atypical keratinocytes [41]. It should be considered the treatment of choice for patients with only a few lesions (about 1-6 lesions) or isolated lesions, or for patients who are non-compliant with topical agents. Cryotherapy is an effective and well-tolerated treatment; it is cheap, rapid and easy to perform, and its efficacy does not depend by patient compliance. However, to date, no standard protocols regarding the time of application of cryogen and the duration of freezing-thawing cycles are available. Olsen's I-II AKs are generally treated with a freeze time between 5 and 20s and one freeze-thaw cycle; whereas in larger and more hypertrophic lesions longer freeze time should be required. Polypodium Leucotomos (PL) is a tropical fern indigenous whose extracts exhibit powerful photoprotective properties after its administration, either topically or orally [42]. *In vitro* and *in vivo* studies demonstrated it prevents damage to the DNA [43,44], lipid peroxidation, activation of pro-inflammatory factors and inhibition of the generation and release of Reactive Oxygen Species (ROS) [45], which damage cellular membranes and induces activation of different Isoforms of Nitric Oxide Synthase (iNOS) [46]. UV-induction of iNOS seems to mediate VEGF-induced angiogenesis and hyperpermeability. PLs' effect also determines inhibition of UV-mediated loss of cell-extracellular matrix adhesion, actin disarray and prevents keratinocyte apoptosis [47]. The MP product we used in our trial contains also a 3-component mixture of DNA-repairing enzymes (photolyase, endonuclease and glycosylase). So far, this product is the only available on the market containing this specific mix of DNA-repairing enzymes. The activity of DNA repairing enzymes when administered topically has been supported by different published studies [48,49]. As recently stated [50], sunscreens containing DNA repair enzymes and antioxidants in addition to the Sun Protection Factor (SPF) can considered as a kind of "medicalized photoprotection" or "active photoprotection reversing the UV-induced specific damage to keratinocyte DNA [51]. Our study demonstrated the benefits of use of an active photoprotection in AK subjects treated with cryotherapy, on the evolution of AKASI score, and LC-OCT features change after therapy. In fact, our study has shown the clinical efficacy and the positive morphological effects of a specific photoprotection product containing PLE, DNA-repairing enzymes and sunscreen filter. The results of this trial suggest that this preventive approach in AK subject treated with cryotherapy could be

relevant both for the prevention of photo-carcinogenesis and its morbidity and for the possibility of reducing the financial burden associated with the treatment, reducing the impact on healthcare costs. Some study limitations should be considered evaluating the results of our trial. First, this was an open not double-blind controlled trial. However, to increase the internal validity of the present study, we performed an assessor-blinded evaluation of the primary outcome (the evolution of AKASI score). In addition, we use an objective non-invasive skin evaluation tool (LC-OTC) to assess the evolution of the cancerization field before and after a MP approach. Another relevant aspect is that the control group is represented by subjects not performing cryotherapy. However, cryotherapy is a lesion-directed therapeutic approach without any relevant effect on field cancerization modification. Therefore, even if the control group was represented by subjects not treated with cryotherapy, we believed that this group could be a significant and adequate comparison in view of the fact that the trial outcomes referred to modification of field cancerization parameters. In our knowledge, this is the first trial evaluating specific sunscreen strategies in AK subjects with this diagnostic approach. Future, randomized, long-term, large sample size, double-blind trials are warranted to better define the protective role of this approach in subjects with severe actinic damage.

CONCLUSION

Prolonged sun exposure is a definite risk factor for the development of skin cancer and actinic keratosis. This is link to the increase DNA damage at keratinocytes level and to the increase oxidative stress caused by UVB and UVA. Sun radiation is also involved in triggering inflammation pathway like the inflammasome. Actinic Keratoses (AKs) are skin lesions deriving from clonal DNA alterations of intraepidermal keratinocytes, determining the development of varying degrees of dysplasia. AK are lesions strictly linked to prolonged and unprotected sun exposure. From a clinical point of view AK are relevant lesions because they can be transformed in squamous cell carcinoma, a potential severe skin cancer. An effective sun protection strategy should address to reduce the oxidative stress at skin level and to reduce the DNA damage. Sunscreens containing DNA repair enzymes and antioxidants in addition to the Sun Protection Factor (SPF) can considered as a kind of “medicalized photoprotection” or “active photoprotection reversing the UV-induced specific damage to keratinocyte DNA. Polypodium Leucotomos Extract (PLE) has well-known antioxidant, photoprotective, and immune-modulatory activities. In addition, some data suggest that PLE administered topically or systemically can increase the efficiency of endogenous DNA-repairing systems. The present study has demonstrated that PLE extract-based photoprotection significantly improves AKASI score in subjects after cryotherapy treatment in comparison with controls. In addition, our study showed that morphological features identified by LC-OCT could be potentially useful for the follow-up of AK subjects.

CONFLICT OF INTEREST

Massimo Milani is an employee of Cantabria labs Difa Cooper.

FINANCIAL SUPPORT

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AUTHOR'S CONTRIBUTION

Pietro Rubegni and Massimo Milani designed the study protocol. Elisa Cinotti performed LC-OCT examination. Arianna Lamberti, Alessandra Cartocci, Carolina Donelli, Giulio Cortonesi and Emanuele Trovato selected the subjects, performed the clinical examinations and the AKASI determination.

SUMMARY STATEMENT

In this study we demonstrated that a medicalized photoprotection strategy (a sunscreen containing potent antioxidant natural extract of Polypodium Leucotomos and a mixture of DNA-reparing enzymes) in subjects with Actinic Keratosis treated with cryotherapy is able to improve in the medium term the clinical evolution assessed with AKASI score. This clinical improvement was also associated with normalization of skin parameters evaluated by non-invasive techniques (Line-field confocal tomography).

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