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Innovative treatments in dermato-oncology: calcium electroporation and daylight photodynamic therapy

Summary of the Ph.D. thesis

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1. INTRODUCTION

The treatment of dermato-oncology patients has undergone significant changes over the last decade. A range of new agents were added to the therapeutic palette, targeting the whole spectrum of skin related malignancies, from precancerous conditions to metastatic form of skin cancers. New therapeutic interventions have also emerged, and some methods have undergone incremental innovation. The changes affect almost all skin tumour types, from the most common keratinocyte-derived skin tumours to malignant melanoma (MM), and also include rare skin malignancies, like merkel cell carcinoma (MCC).

Electrochemotherapy (ECT) is a treatment option for primary and metastatic solid tumours. The procedure uses electrodes to place the tumour in an electric field and delivers a cytostatic agent (bleomycin) across the cell membrane to the cells. ECT targets the mitotically active cells, and selectively destroys the tumour cells and the endothelial cells of the supplying blood vessels, while the surrounding tissues remain intact. ECT has non-thermal tumour ablative, antihemorrhagic and antiangiogenic effects. During calcium electroporation (EP) the intratumoral administration of calcium is followed by electrical pulses on the tumour. The properties of ECT and Ca-EP are very similar, therefore calcium may replace bleomycin during electroporation treatments.

Photodynamic therapy (PDT) is a well recognized therapy in guidelines for actinic keratosis (AK), superficial basal cell carcinoma (BCC) and Bowen's disease. It is a non invasive medical treatment, which is based on the activation of light-sensitive molecules (photosensitizers) in the diseased tissues, resulting in the formation of reactive oxygen species, and leading to injury and cell death. Depending on the light source, PDT can be performed using artificial light or natural sunlight. The former is called conventional photodynamic therapy (c-PDT), while the latter daylight photodynamic therapy (d-PDT). The efficacy of d-PDT and c-PDT in the treatment of both superficial BCC and AK is similar, but d-PDT is better tolerated and is associated with significantly less pain.

The use of ECT and PDT in the treatment of skin tumours started decades ago and has undergone significant incremental innovation, which continues today. In Hungary, both methods were first introduced at the Department of Dermatology and Allergology in Szeged (PDT: 2003, ECT: 2007), and are part of routine dermato-oncological care. The scientific work underlying this thesis presents the results obtained with the innovative versions of the two methods, calcium electroporation and daylight photodynamic therapy.

2. AIMS

2.1. Calcium electroporation in the treatment of cutaneous metastases

The primary objective of the study was to compare the efficacy of bleomycin-based ECT and Ca-EP in the treatment of cutaneous metastases of malignant tumours. Secondary objective was to register adverse events (AE/SAE) during Ca-EP and bleomycin-based ECT.

2.2. Daylight photodynamic therapy in the treatment of actinic keratoses

The primary aim of the study was to confirme the efficacy of d-PDT in the treatment of actinic keratoses under the local climatic conditions. Secondary objectives were to study the safety and tolerability of d-PDT in the treatment of AK under the local climatic conditions, with particular focus on treatment-related pain. Tertiary objectives were to adapt a treatment protocol of d-PDT in the treatment of AK and introduce the method in our department.

3. PATIENTS AND METHODS

3.1. Calcium electroporation in the treatment of cutaneous metastases

3.1.1. Design of the clinical trial

The study protocol was approved and registered by the Centre for Health Registration and Education on 3rd of May 2016 under case number 032104/2016/OTIG. Our clinical trial was performed at the Department of Dermatology and Allergology, University of Szeged, with the permission of the Institutional and Regional Research Ethics Committee of Human Biomedical Sciences, University of Szeged (clinicaltrials.gov number: NCT03628417, registration date: 23 May 2016; permission number: 3806; registration number: 98/2016-SZTE; protocol number: ECT-KALCIUM-001).

3.1.2. Patients and methods

Patients enrolled in the study had at least one histologically confirmed metastasis of 0.5 to 3 cm in size that was accessible to electroporation. Patient inclusion criteria were as follows: age > 18 years, ECOG status ≤ 2, life expectancy more than 3 months, platelet count ≥ 50 billion/L, international normalised ratio (INR) international normalised ratio (INR) < 1.5 and a period of more than 2 weeks without treatment. Only medical cancer treatments (endocrine treatment, targeted treatment and radiotherapy to another area) were allowed. If there was no regression of cutaneous metastases, the continuation of vinorelbine, capecitabine or

paclitaxel therapies were allowed. Patients were excluded from the trial if they had severe allergic reactions associated with bleomycin or if they previously received a dose of bleomycin that was more than 200,000 units/m2. Pregnancy, lactation, untreated coagulation disorders were also reasons for exclusion. Previously irradiated cutaneous metastases and concomitant treatments were recorded. The intervention was performed after oncological recommendation.

A maximum of 10 cutaneous or subcutaneous metastases from 0.5-3 cm of any histological type were included per patient. One to 6 metastases (depending on the patient's number of metastases) were numbered 1-6 and randomised (ratio 1:1) into one of two treatment arms (Ca-EP, Bleomycin-based ECT). The treatment of the remaining metastases (6-10) was known and these were used for biopsy sampling without evaluation of their clinical response. Randomization of metastases for each patient was performed using the nQuery Advisor 7.0 computer program. Randomization and blinding was performed by an independent clinical pharmacologist, who prepared and labelled syringes containing clear material according to the numbered metastases. The randomisation code was revealed 6 months after treatment.

The concentration of the prepared solutions were ations used 220 mmol/l (9 mg/ml) for calcium chloride (based on preclinical data), 1000 IU/ml for bleomycin (according to ESOPE guidelines). The tumour volume was determined with the following formula: $ab^2/6$ ("a" = maximum diameter, "b" = maximum diameter perpendicular to "a"). The amount of drug injected into the tumour was based on the tumour volume (tumour < 0.5 cm^3 - 1 ml/cm^3 tumour volume, tumour > 0.5 cm^3 - 0.5 ml/cm^3 tumour volume). The electrical pulses were delivered by a Cliniporator (IGEA, Carpy, Italy) according to the European Standard Operating Procedures on Electrochemotherapy (ESOPE) guidelines. Linear needle electrodes (8 pulses 400 V and 1000 V/cm, 0.1 ms duration, 5 kHz frequency) and hexagonal needle electrodes (4 pulses 730 V and 910 V/cm, 0.1 ms duration, 5 kHz frequency) were used according to the tumour location and size. The single procedure was performed under local or general anaesthesia after a prior doctor-patient consultation.

3.1.3. Assessment of treatment outcomes and adverse events

The clinical study lasted 12 months. Tumour response at each follow-up visit (1-7, 15, 30, 60, 90, 180 and 360 days after treatment) was assessed according to the WHO RECIST 1.1 criteria and photo-documentation was performed. Patients' Quality of Life (QOL) was assessed before and after treatment (0-100%). Pain was measured using the Numeric Rating Scale (NRS) (0-10 points: 0 = no pain, 1-3 = mild pain, 4-6 = moderate pain, 7-10 = severe

pain). Potential adverse events were recorded using the Common Toxicity Criteria for Adverse Events version 4.0 (CTCA version 4.0). Biopsies were performed from the tumour area before treatment and/or 7 days after treatment at patients with more than 6 (6-10) metastases. The randomization code was revealed six months after treatment and biopsies were taken from both calcium and bleomycin-treated lesions and analysed by our clinic's histopathologists for the presence of residual tumour tissue, inflammation, fibrosis and necrosis.

3.1.4. Statistical analysis

Statistical analyses were performed using IBM SPSS, v24, software and R statistical software. Objective tumour response (OR) was assessed using Fisher's exact test 6 months after treatment. The required number of tumours included in the clinical trial was determined by a non-inferiority test, with a minimum of 28 evaluable tumours at a significance level of 0.05 and a power of 80%. A non-inferiority cut-off of 20% was used to determine the clinical difference between the two treatment arms.

3.2. Daylight photodynamic therapy in the treatment of actinic keratoses

3.2.1. Design of the clinical trials

Our clinical trials with d-PDT were performed at the Department of Dermatology and Allergology, University of Szeged, with the approval and permission obtained from the Institutional and Regional Research Ethics Committee of Human Biomedical Sciences, University of Szeged (protocol: PDT-DLIGHT; registration date: 04.11.2014; registration no: 137/2014). A total of three clinical trials with d-PDT (PDT-DLIGHT-001, -002, -003) were conducted. Inclusion and exclusion criteria, assessment of treatment outcomes and adverse events were similar in all three trials. The treatment protocol in all studies was identical in the four major steps. Differences between studies were in the incubation time of the photosensitizer, and the duration of time patient spent under natural sunlight. The treatment parameters suggested by the international and European protocol were used as a basis, and changed step by step in order to optimize it under the local climatic conditions (Wiegell *et al.* JEADV, 2012; Morton *et al.* JEADV, 2015).

3.2.2. Patients and methods

In the studies with d-PDT we included patients who were older than 18 years. Other inclusion criteria were AK localised to the head and neck region, and the diameter larger than 6 mm.

In our clinical trials, we primarily recruited patients presenting with a single AK. If there were multiple AKs, we assigned a target lesion, which was followed in the clinical trials for response and adverse events. The diagnosis of AK was based on the clinical picture and on the dermoscopic presentation. Histopathological verification of the skin lesions was performed only in selected cases. We excluded from the studies those patients, who underwent medical intervention (cryotherapy, topical medication, surgical or laser treatment, radiotherapy) in the area of the AK six weeks prior to the study, and who had known hypersensitivity to the photosensitizing agent. After receiving detailed information on the procedure and purpose of the trial, patients confirmed their willingness to participate in the study by signing a consent form. Photodynamic therapy with natural sunlight was performed in four steps. The skin area to be treated was prepared according to the international protocol [46, 51, 52]. Hyperkeratosis was removed from the AK with a Volkmann cannula. A high factor sunscreen was applied to the AK, and to the surrounding skin. Ten percent 5-aminolevulinic acid (ALA) was used as a photosensitizer in a magistral cream, and applied without occlusion to the AK and its 5 mm surrounding area. Light intensity was measured in the PDT-DLIGH-002 and 003 trials with the Vector H410 dosimeter (Scientech Inc. Boulder, CO, USA). The following formula was used to calculate the treatment dose or the time patients spent under daylight: dose (J/cm^2) = [light intensity (mW/cm^2) × treatment time (min) × 0.6]/10. The d-PDT treatment was postponed in case of rainy wheater conditions.

3.2.3. Assessment of treatment outcomes and adverse events

Clinical response was assessed based on the clinical and dermoscopic appearance six weeks after treatment at the follow-up visit. Complete remission (CR) was considered, if the tumour completely regressed, while a partial remission (PR) if at least one third of the lesion regressed. Progressive disease (PD) was defined as an increase in the size of the AK, while stable disease (SD) was defined when regression was seen in less than one-third of the lesion. Adverse events were recorded using the CTCA version 4.0. Severity of AEs was rated according to a 5 point scale (0-4 points: 0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe). Pain during treatment was measured on a visual analogue scale (VAS, scores 0-10: 0 = no pain, 10 = unbearable pain).

3.2.4. Statistical analysis

Statistical analysis in the d-PDT trials, as well as the comparison with the previous trial with c-PDT, were performed using IBM SPSS 26 software (IBM Corporation, Armonk, NY, USA). Data were not found to be normally distributed by one-sample Shapiro-Wilk test.

Chi-square test was used to analyse the efficacy of PDT, and Kruskal-Wallis test to compare pain scores. Pairwise comparisons were performed using Dunn's post-hoc test. Comparisons of complete remission rates after the significant chi-squared test were performed using Benjamini-Hochberg correction. A threshold level of five percent was considered to indicate statistical significance.

4. RESULTS

4.1. Calcium electroporation in the treatment of cutaneous metastases

4.1.1. Evaluation of clinical response

A double-blinded randomised controlled trial was conducted in our institute to compare the efficacy of Ca-EP with bleomycin-based ECT in skin metastases (ClinicalTrials.gov: NCT03628417). Seven patients (5 women, 2 men) with a total of 44 cutaneous metastases (34 malignant melanoma, 10 breast cancer) were included in the clinical trial between October 2016 and June 2018 (Figure 1.).

Six patients with MM were treated for cutaneous metastases located on the lower extremity, while one patient was treated for breast cancer metastases localised to the trunk. The median age of the patients was 73 years (interquartile range: IQR = 21). 33 of the 44 metastases were randomised to the two treatment arms and their clinical response was evaluated, while 11 lesions were histologically analysed (Figure 2.). Of the randomised metastases, 18 were treated with Ca-EP (15 MM, 3 breast cancer) and 15 with bleomycin-based ECT (12 MM, 3 breast cancer). Of the 33 randomised cutaneous metastases, 6 (18%) were located in previously irradiated areas (2 lesions treated with Ca-EP, 4 lesions treated with bleomycin-based ECT). The median value of the maximum diameter of the 33 metastases evaluated by clinical response was 7 mm (IQR = 5). The median injected dose was 0.0855 mL (IQR = 0.1924) for Ca-EP and 0.132 mL (IQR = 0.27) for bleomycin-based ECT. Four of the procedures were performed under local anaesthesia and 3 under general anaesthesia. Hexagonal needle electrodes were used for 21 (63.6%) and linear for 12 (36.4%) metastasis during EP.

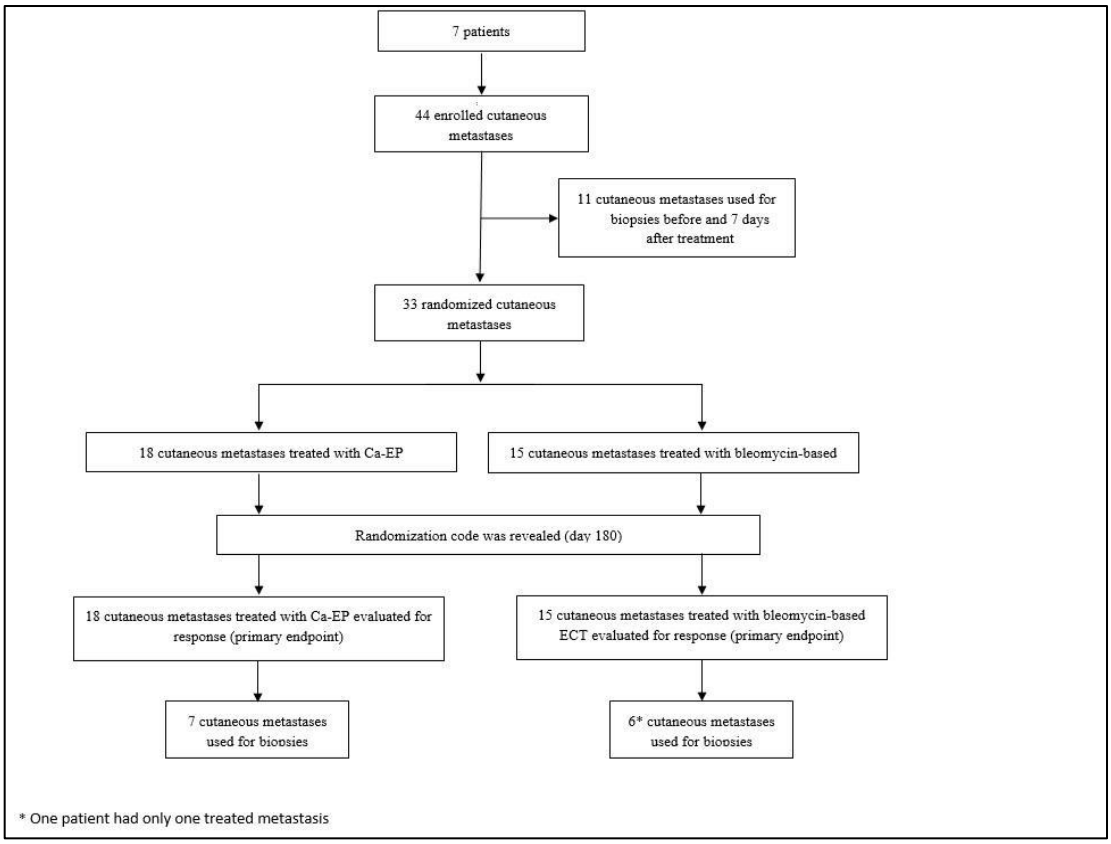

Figure 1. Illustration of trial profile.

Of the 18 lesions treated with Ca-EP, hexagonal electrodes were used for 11 (61.1%) and linear electrodes for 7 (38.9%). Of the 15 lesions treated with bleomycin-based ECT hexagonal electrodes were used for 10 (66.7%) and linear for 5 (33.3%) (Table 1.).

The OR for Ca-EP was 33% (CR = 22%; PR = 11%) and for bleomycin-based ECT was 53% (CR = 40%; PR = 13%). There was no significant difference in either OR ($p = 0.30$) or CR ($p = 0.45$) between the two treatment arms.

After six months, 33% (6 out of 18) of metastases had progressed in calcium-treated lesions compared to 13% (2/15) of bleomycin-treated lesions. There was no significant difference in the clinical response of lesions located in previously irradiated areas compared to other cutaneous metastases ($p = 0.37$). Before and 7 days after treatment, 11 cutaneous biopsies were taken from the cutaneous metastases.). Out of the 13 histological samples taken 6 months after treatment, 6 (3 lesions treated with Ca-EP, 3 lesions treated with bleomycin-based ECT) showed clinical CR, based on the randomization code. In 5 of the 6 cases, no tumour cells were detected, so that CR could be confirmed by histopathological examination (3 lesions treated with Ca-EP and 2 lesions treated with bleomycin-based ECT).

Treatment arm	Calcium electroporation			Bleomycin-based electrochemotherapy		
	Our trial	**2018 Study**	**Total**	**Our trial**	**2018 Study**	**Total**
Tumour size						
Median of the largest diameter, mm	6-5 (5-30)	9-5 (5-18)		7 (5-25)	11 (4-25)	
Tumour type						
Malignant melanoma	15	1	16	12	1	13
Breast cancer	3	17	20	3	18	21
Previously irradiated lesions, n	2	8	10	4	7	11
Location						
Lower extremity	15	4	19	12	4	16
Trunk	3	14	17	0	15	15
Upper extremity	0	0	0	3	0	3
Treatment						
Median doses (range), mL	0-085 (0,042-3,14)	0-24 (0,03-1,21)		0,132 (0,065-0,475)	0,21 (0,03-0,55)	
Median delivered current (range), A	3,85 (1,4-9)	3-4 (0,9-8,2)		4 (1,4-6,5)	2,8 (1-9,6)	
~ with linear electrodes (range), A	4 (2,25-9)	3-4 (0,9-8,3)		5,05 (4-6,1)	2,8 (1-9,6)	
~ with hexagonal electrodes (range), A	2-5 (1,4-4,2)	NA		2,75 (1,4-3,6)	NA	
Median number of applications (range), n	1 (1-6)	3 (1-7)		1 (1-3)	3 (1-7)	
Electrodes						
Linear	39% (7)	100% (18)		33% (5)	100% (19)	
CR for linear electrode subgroup	14% (1)	66% (12)		0	68% (13)	
Hexagonal	61% (11)	0		67% (10)	0	
CR for hexagonal electrodes subgroup	27% (3)	NA		60% (6)	NA	
Clinical response						
Complete response, percent, n	4	12	44-44% (16)	6	13	55-88% (19)
Partial response, percent, n	2	1	8-33% (3)	2	3	14-7% (5)
Stable disease, percent, n	6	3	25% (9)	5	0	14-7% (5)
Progressive disease, percent, n	6	2	22-22% (8)	2	3	14-7% (5)
Adverse events						
Ulceration, percent, n	2	7	25% (9)	3	13	47-05% (16)
Itch, percent, n	0	1	2-77% (1)	0	5	14-7% (5)
Hyperpigmentation, percent, n	2	0	5-55% (2)	6	5	32-35% (11)
Exuding, percent, n	0	2	5-55% (2)	0	2	5-88% (2)

Table 1. Results of our clinical trial and comparison with the first phase II randomised double-blind trial by Falk *et al.* in 2018.

4.1.2. Summary of the physical parameters of the treatment

Differences were observed in tumour response and electrode type between the two treatment arms, which were not statistically significant due to the small sample size. OR values were higher in Ca-EP with linear electrodes (42.8%, 3/7) than with hexagonal electrodes (27.3%, 3/11) ($p = 0.63$). 45.45% (5/11) of tumours classified as PD were treated with hexagonal electrodes and 14.29% (1/7) with linear electrodes ($p = 0.32$). The opposite trend was observed with bleomycin-based ECT. For lesions with OR, 70% (7/10) were treated with

hexagonal electrodes and 20% (1/5) with linear electrodes (p = 0.12). 10% (1/10) of tumours classified as PD were treated with hexagonal *electrodes* and 20% (1/5) with linear electrodes (p = 1). There was no significant difference in the delivered currents between the two treatment arms (p = 0.956), nor between metastases treated in previously irradiated and non-irradiated areas (p = 0.911).

4.1.3. Adverse events during treatment

No serious adverse events were observed. Based on the CTCA 4.0 criteria system, grade I adverse events such as ulceration and hyperpigmentation after Ca-EP were observed in 2-2 metastases (2/18, 11%). With bleomycin-based ECT, ulceration occurred in 20% (3/15) and hyperpigmentation in 40% (6/15) of treated lesions. The median NRS score for pain before treatment was 2 (IQR = 2), immediately after treatment 2 (IQR = 9), with 3 patients reporting no pain (NRS: 0), 1 reporting mild pain (NRS: 1-3) and 1 reporting moderate pain (NRS: 4-6). Two patients with more than 6 cutaneous metastases who underwent biopsy reported severe pain (NRS: 9-10). The median 6-month NRS score was 2 (IQR = 4), with a mean Ca-EP of 2.5 (σ = 3.2016) and 4.5 (σ = 2.2913) for patients treated with ECT. At six months after treatment, QOL scores were the same or increased from baseline from 70% (IQR = 10) to 80% (IQR = 10). Three patients experienced no change in their quality of life at 6 months after treatment. All 7 patients agreed to repeat treatment in the future if necessary.

4.1.4. Follow-up data

The mean follow-up of 6 patients lasted 29 months (standard deviation = 6.8232). One patient died 11 months after treatment (due to progression of another known primary tumour) and two patients died 26 and 27 months after treatment due to femoral neck fracture and malignant progression of melanoma, respectively. None of the lesions showing CR recurred during the 1-year follow-up period.

4.2. Daylight photodynamic therapy in the treatment of actinic keratoses

4.2.1. Evaluation of treatment outcomes and adverse events

In the PDT-DLIGHT-001 study, the photosensitizer was incubated without occlusion on the skin for 30 minutes according to the international protocol (Table 2.). Afterwards, patients were exposed to sunlight for 90 minutes. 63 patients (33 male, 30 female) with AK (grade I) were treated in the head and neck region (face, forehead, nose). The average age of the patients was 75.37 years (range 49-92 years). One hour before treatment, the mean outdoor

temperature was 25.63°C (12-32°C). Treatment was performed under cloudy (1-13 °C), partly sunny (10-25 °C) and sunny (17-31 °C) weather conditions. 73% of AKs (n = 46) showed complete, and 27% (n = 17) partial remission. Pain during treatment was rated by the patients as 0.3 (0-5) on the VAS. In the PDT-DLIGHT-001 study, we observed that spending 90 minutes outdoors under sunny and partly sunny weather conditions was difficult to tolerate for our elderly patients. Taking this into account, in the PDT-DLIGHT-002 study we increased the duration of the incubation time of the photosensitizer, which patients spent indoors, while shortened the treatment time outdoors. After application of the photosensitizer cream, we waited 120 minutes instead of 30 to allow photoporphyrin (Pp) IX accumulation. Patients stayed indoor during the incubation period (9-11 a.m.) and then were exposed to natural sunlight for 30 minutes. The treatments took place under cloudy (18-29°C), partly sunny (19°C) and sunny (25-35°C) weather conditions. The PDT-DLIGHT-002 study included 30 patients (16 men, 14 women) with an average age of 77.9 years (41-97 years). The average natural sunlight intensity measured with a dosimeter (Vector H410) prior to treatment was 48.63 mW/cm² (7-71 mW/cm²). Patients spent 30 minutes outdoors and the average treatment dose was 87.5 J/cm² (12.6-127.8 J/cm²) (Table 2.).

Patient and treatment characteristics, results	PDT-DLIGHT			c-PDT Gaál et al.
	001	002	003	
Number of patients (n)	63	30	73	22
Sex (male to female) (n)	33:30	16:14	47:26	11:11
Average age (years)	75,37 (49-92)	77,90 (41-97)	74,64 (51-92)	75,86 (62-92)
Photosensitizing agent	10% ALA			20% ALA
Indoor incubation time (min)	30	120	120	240
Outdoor treatment time (min)	90	30	10,42 (2-60)	12 min/field
Measured brightness (mW/cm²)	NA	48,63 (7-71)	46,67 (2-92)	NA
Treatment dose (J/cm²)	NA	87,5 (12,6-127,8)	19,47 (7,2-54)	37
Complete Remission % (n)	73,01 (46)	63,33 (19)	84,93 (62)	59,09 (13)
Partial Remission % (n)	26,98 (17)	36,66 (11)	15,07 (11)	31,82 (7)
Stable disease % (n)	0	0	0	9,09 (2)
Progressive disease % (n)	0	0	0	0
Pain (VAS scale: 0-10)	0,3 (0-5)	2,13 (0-10)	0	6,94 (3-10)
Patients under pain relief (n)	0	0	0	21

Table 2. Patient characteristics, treatment parameters and outcomes in studies with d-PDT and c-PDT by Gaál *et al*. (Acta Derm Venereol, 2012).

Treatment efficacy was evaluated 6 weeks after treatment. 63.33% (n = 19) of AKs showed CR and 36.66% (n = 11) PR. Patients tolerated the therapy well, with a mean pain score of

2.13 on the VAS scale. The pain was mild, resolved spontaneously, and no treatment interruption was necessary. 33% of patients (n=10) experienced severe erythema within 24 hours of treatment and presented in our department. With the use of topical skin soothing treatment, the erythema subsided within a few days, then disappeared completely. We found that these patients were treated under sunny weather conditions at higher doses (above 100 J/cm²). In our study, lesions treated with doses below and above 15 J/cm^2 showed no difference in clinical responses (CR and PR). The PDT-DLIGHT-003 clinical trial used dosimetry to measure light intensity. Considering the severe erythema seen previously at doses above 100 mJ/cm^2, we aimed to use lower treatment doses. We included 73 patients (47 males, 26 females) with a mean age of 74.64 years (51-92 years). The mean light intensity was 46.67 mW/cm² (2-92 mW/cm²). The mean outdoor treatment time was 10.42 min (2-60 min). During treatment, patients received an average dose of 19.47 J/cm² (7.2-54 J/cm²). There was no pain or severe erythema during the treatment. At the 6-week follow-up, 85% (n = 62) of the treated AKs showed CR and 15% (n = 11) PR.

4.2.2. Comparing conventional and daylight photodynamic therapy

We compared the results of our studies with d-PDT and the investigations with c-PDT by Gaál *et al*. (Acta Derm Venereol, 2012). There was no significant difference in efficacy between treatments based on the international d-PDT protocol (PDT-DLIGHT-001) and the modified protocol (PDT-DLIGHT-003) ($p = 1$). D-PDT (PDT-DLIGHT-003) was significantly more effective compared to c-PDT ($p = 0.003$, khi-square test). The khi-square test showed a significant difference in CR rates between the four groups ($p=0.003$), with the PDT-DLIGHT-003 protocol leading to the highest proportion (84.9%) of patients reaching CR. A significant chi-squared test for comparison of CR rates showed a significant difference ($p = 0.045$) between the PDT-DLIGHT-003 protocol (84.9%) and the c-PDT protocol (59.1%) with Benjamini-Hochberg correction, favouring d-PDT. Statistical analysis showed that there was a significant difference between the pain scores in the four groups ($p < 0.001$, Kruskal-Wallis test). Dunn's post-hoc analysis showed that the pain scores were significantly higher ($p < 0.001$) with c-PDT (Gaál M. *et al*.), compared to d-PDT.

4.2.3. Adaptation of the international treatment protocol of daylight photodynamic therapy

For our elderly patients the d-PDT was difficult to tolerate in the PDT-DLIGHT-001 trial, because they had to spend 90 minutes under natural daylight. We modified the proportion of time spent indoors and outdoors, while the overall length of treatment did not change

significantly (120 versus 150 min). In the PDT-DLIGHT-002 trial we achieved similar efficacy with the modified protocol, CR in two third and PR in one third of AKs. However, d-PDT with a 30 min outdoor treatment time resulted in adverse events. 30% of our patients presented to our department with severe erythema within 24 hours of treatment. Analyses showed, that severe erythema occurred in patients receiving doses above 100 J/cm². Our protocol was further modified in order to avoid high treatment doses. We incorporated dosimetry to measure the light intensity and calculated the treatment time patients needed to spend outdoore. In our subsequent study (PDT-DLIGHT-003), the average treatment dose was around 20 J/cm² and the average treatment time around 10 minutes. We observed CR in 85% of AKs and PR in 15%. Based on previous protocols and on our own findings, after step by step modification we have developed a local protocol for d-PDT in Szeged and a detailed explanation with pictures about the procedure for patients. We introduced the local protocol for d-PDT in AK into the daily dermatological practice at our departement.

5. DISCUSSION

5.1. Calcium electroporation in the treatment of cutaneous metastases

A double-blind, randomized, controlled trial was conducted at our department between October 2016 and June 2018 to compare the efficacy of Ca-EP and bleomycin-based ECT in skin metastases in 7 patients. This was the second clinical trial evaluating the efficacy of Ca-EP. Patients included in our study were treated for at least one, and up to 10, histologically confirmed malignant melanoma or breast cancer metastases of 0.5-3 cm in size. After randomization, patients received either calcium or bleomycin intratumorally, followed by reversible EP. Six months after treatment, based on clinical and histological tumour response assessment, Ca-EP achieved comparable efficacy to bleomycin-based ECT in eradicating tumours, with a better side effect profile. CR after Ca-EP was also confirmed by histological examination.

Seven patients with a total of 44 skin metastases (34 MM, 10 breast cancer) were included in the trial. Eleven metastases were taken for biopsy, 33 metastases were randomized and treated once. The OR was 33% (6/18) for Ca-EP and 53% (8/15) for bleomycin-based ECT, with overall response rates of 22% (4/18) and 40% (6/15). CR was confirmed by histopathological examination in both arms. No serious adverse events were recorded. Grade I adverse events such as ulceration and hyperpigmentation were more frequent during bleomycin-based ECT than after Ca-EP. The effect of Ca-EP was "non-inferior" compared

to bleomycin-based ECT. Our results suggest that Ca-EP is an effective and safe procedure for the treatment of cutaneous metastases located on the skin.

In the first clinical trial with Ca-EP in Denmark, 7 patients (6 breast cancer and 1 MM) were treated for skin metastases (Falk *et al.*, Acta Oncol. 2018). There was no significant difference in OR between Ca-EP, 72% (66% CR) and ECT 84% (68% CR). In this study, Ca-EP proved to be "non-inferior" and only mild side effects were observed with both treatment arms, such as ulceration of the treated area similar to those observed in our study (Table 1.). The higher response rate in the Danish study could be explained by the different histological type of the treated skin lesions and by the use of different electrodes. In our own study, we treated mainly MM metastases (81.8%, n = 27), whereas in the Danish study the number of MM metastases was low (5.4%, n = 2). In our clinical trial, 89% (24/27) of the treated MM metastases were BRAFV600E wild-type (WT). One study found bleomycin-based ECT to be more effective in melanoma showing BRAFV600E mutations compared with BRAF-WT tumours (Rolinsek, Radiol Oncol, 2016). This difference may explain at least partially the lower response rates compared to previous results, but requires further investigations on molecular level.

Another difference between the two clinical trials was the type of electrodes used. In our study, mainly (63.6%) hexagonal electrodes were used, whereas in the first study only linear electrodes. The electric field distribution is known to differ between linear (smaller diameter) and hexagonal electrodes. For this reason, the field distribution is more symmetric and less cold spots are observed with linear electrodes. Although no significant difference was observed due to the small number of cases, Ca-EP was more effective with linear electrodes ($p = 0.30$). In preclinical studies, increasing the electric field up to 0.8-1.0 kV/cm during Ca-EP significantly decreased ATP levels and the cell viability [35, 42]. This may explain the observed discrepancy, as the electric field was 1000 V/cm with linear and 910 V/cm with hexagonal electrodes. Further studies are needed to fully explore this issue. There was no significant difference in the current delivered between the two treatment arms in any of the clinical trials. The difference may be more relevant in the treatment of large tumours. We observed grade I local AEs in both treatment arms, no grage 3-4 AEs have been recorded. Ulceration and hyperpigmentation occurred more frequently after bleomycin-based ECT (20% and 40%) than during Ca-EP (both 11%). Our observations were similar to the results of the first study, ulceration during Ca-EP affected only the tumour region, sparing the surrounding normal skin. None of the lesions treated with Ca-EP in the first study showed hyperpigmentation, which may be related to the exclusive use of linear electrodes.

Limitations of our study include the low number of metastases evaluated and the fact, that different electrodes (hexagonal or linear) were used for the treatments.

Ca-EP has a similar adverse event profile to bleomycin-based ECT, with the difference that the flu-like symptoms associated with chemotherapeutic agents do not occur with Ca-EP, and the treated skin rashes are less hyperpigmented. When the electrical pulses are delivered, there may be brief contractions in the muscles underlying the treated area. With local anaesthesia, muscle contractions may be uncomfortable. The treated area may show erythema, oedema, and rarely necrosis, which heals in 6-10 weeks. Painkillers, and incase of superinfection antibiotics can be used. The most commonly reported side effects of bleomycin-based ECT are post-operative pain (10%) and flu-like symptoms (10%). The latter is due to bleomycin, and is not expected with Ca-EP. Hyperpigmentation during bleomycin-based ECT occurred in 7% of cases, while after pooling the results of the first two clinical trials, it occurred in 5.55% of cases during Ca-EP.

5.2. Daylight photodynamic therapy in the treatment of actinic keratoses

Actinic keratoses may progress towards invasive SCC. It is known, that 60-80% of invasive SCCs arise from AK. Therefore treatment of AK is of high importance, and PDT is a promising option. Compared to conventional PDT, daylight PDT is a simplified treatment procedure. In c-PDT, the photosensitizer is incubated on the skin for three hours in occlusion, while in d-PDT it is incubated for 30 minutes without occlusion. Another important difference is, that d-PDT uses the natural sunlight for the excitation of the photosensitizer, while c-PDT an artificial light source. Probably the major advantage compared to c-PDT is, that there is significantly less pain associated with d-PDT. The international protocol for d-PDT treatment is based on clinical trials conducted in centres mostly located in Northern Europe. As d-PDT is performed in the open air and weather conditions may affect the feasibility, efficacy and tolerability of the treatment, it is necessary to adapt the protocol of the method to the local climatic conditions.

The aims of our work were to study the efficacy, safety and tolerability of d-PDT for the treatment of AK, and to introduce the intervention in our department. We used the international protocol of d-PDT and modified it step by step according to the results obtained during clinical trials under local climatic conditions. We conducted three clinical trials with d-PDT. We started to use the international protocol and based on tolerability, we modified first the the proportion of time patients spent indoors (from 30 to 120 min) and outdoors (from 90 to 30 min), while the overall length of treatment did not change significantly. Later,

we introduced dosimetry to avoid high treatment doses. With our final protocol of d-PDT, the average treatment dose was around 20 J/cm² and the average treatment time around 10 minutes. We found that performing d-PDT in AK with the modified protocol - optimizing the time spent outdoors and aiming for lower treatment doses - did not change the efficacy of the intervention, but was better tolerated by the elderly patient population. We also compared results with using d-PDT to previous study results with c-PDT in our department, reported by Gaál M *et al*. (Acta Derm Venereol, 2012). We confirmed previus literature findings, that d-PDT was similarly effective but less painful compared with c-PDT.

Finally we have developed a local protocol for d-PDT in Szeged, and a description about the procedure for patients using pictures. Based on our protocol, the first step of d-PDT is to remove the hyperkeratosis from the skin area to be treated. A high factor sunscreen is recommended to be applied to the treatment area and to the surrounding skin. The treatment involves incubation of an extemporary formulated cream containing 10% ALA on the skin for 120 min without occlusion. The upper threshold of the treatment dose is 100 J/cm^2, while the lower threshold was known to be 8 J/cm^2. In our experience, treatment doses below or above 15 J/cm^2 showed no difference in clinical responses (CR and PR). Therefore, we recommend to use lower treatment doses in d-PDT and avoid doses above 100 J/cm^2. To measure the actual light intensity, we recommend the use of dosimetry. From the light intensity and required treatment dose, it can be calculated how much time patients should spend outdoors. During summer time this time is recommended to keep around 10 min. Based ont the climatic conditions, we found it optimal in our region to carry out d-PDT between May and October. In rainy weather, and if the temperature outside is below 10 °C or above 35 °C, the treatment has to be postponed.

6. CONCLUSIONS

The use of ECT and PDT in the treatment of skin tumours has undergone significant incremental innovations in the past decades. Two examples are the calcium electroporation and the daylight photodynamic therapy. Based on the results and new findings of our resent scientific work, both methods are promising to use in the routine dermato-oncological care.

6.1. Calcium electroporation in the treatment of cutaneous metastases

1. With Ca-EP similar efficacy was achieved in tumour eradication with a better adverse event profile, than with bleomycin-based ECT. Our results confirmed the findings of the first clinical trial, that Ca-EP is "non-inferior" to bleomycin-based ECT in the treatment of cutaneous metastases.
2. Clinical complete remission after Ca-EP was confirmed for the first time by histological examination.
3. Given the growing number of cancers worldwide, Ca-EP may be a new, effective, and safe treatment for skin tumours, especially in patients for whom chemotherapeutic agents are contraindicated.

6.2. Daylight photodynamic therapy in the treatment of actinic keratoses

1. We confirmed, that daylight PDT is effective in the treatment of AK under the local climatic conditions.
2. Daylight PDT in the treatment of AK is safe and well tolerated by our patients. We improved tolerability and safety of the method by reducing the time patient need to spend outdoor and by using dosimetry. The method is related with less pain compared to conventional PDT.
3. D-PDT performed according to the modified international treatment protocol was successfully introduced in our department.

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LIST OF PUBLICATIONS

This doctoral thesis is based on the following publications

I. **Ágoston D,** Baltás E, Ócsai H, Rátkai S, Lázár PGy, Korom I, Varga E, Németh IB, Viharosné Dósa-Rácz É, Gehl J, Oláh J, Kemény L, Kis EG. Evaluation of calcium electroporation for the treatment of cutaneous metastases: a double blinded randomised controlled phase II trial. Cancers. 2020; 12: 179. **IF: 6.639**

II. **Ágoston D**, Hánis Cs, Ócsai H, Csányi I, Varga E, Korom I., Németh I, Kis E, Kemény L, Oláh J, Baltás E. Multimodal treatment options for Merkel cell carcinoma. [A Merkel sejtes karcinóma multimodális kezelési lehetőségei.] Bőrgyógy Vener Szle. 2022; 98: 240-246. [Hungarian]

III. **Ágoston D,** Ócsai H, Ignácz F, Viharosné Dósa-Rácz É, Rárosi F, Oláh J, Kemény L, Baltás E. Innovation in the treatment of non-melanoma skin cancer: daylight photodynamic therapy. [Új eljárás a nem melanoma típusú bőrdaganatok kezelésében: "daylight" fotodinámiás terápia.] Orv Hetil. 2022; 163:36. [Hungarian] **IF: 0.707**

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Publications not directly related to the thesis:

I. Kis E, Baltás E, Ócsai H, Csányi I, Ottlakán A, Lázár Gy, Vass G, **Ágoston D**, Rózsa P, Bottyán K, Dalmády Sz, Nagy A, Tóth-Molnár E, Oláh J. Milestones of electrochemotherapy. [Az elektrokemoterápia mérföldkövei.] Bőrgyógy Vener Szle. 2023; 99: 116–120. [Hungarian]

II. Rózsa P, **Ágoston D**, Szederkényi E, Ócsai H, Baltás E, Vass G, Kemény L, Oláh J, Kis E. Electrochemotherapy for multiple cutaneous tumors in immunosuppressed patients. [Immunszupprimált betegek multiplex bőrdaganatainak elektrokemoterápiás kezelése.] Orv Hetil. 2023; 164: 1462-1468. [Hungarian]