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Combination of cold plasma and pulsed electric fields – A rationale for cancer patients in palliative care

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ABSTRACT

Cancer continues to be a significant threat to human health. Oncotherapy, therefore, relies on the combination of different approaches to increase a patient's chances. If therapeutic options are exhausted, effective palliation can at least still improve the quality of life. Over two decades ago, electrochemotherapy (ECT), which is based on pulsed electric field (PEF) exposures, was introduced in palliative care to alleviate the burden imposed by tumors, such as malignant melanoma and breast cancer. In this case, pulsed electric fields with a duration in the range of microseconds permeabilize cell membranes and permit reducing dosages of cytotoxic drugs and accordingly of associated systemic side effects. More recently, exposures to pulsed electric fields, shorter than the cellular plasma membrane charging time, have been found to affect subcellular structures and cell functions directly, i.e., without additional drugs. Instead of cytotoxic drugs, also the delivery of genes and calcium is currently investigated for alternative treatment options. Another way to induce tumor cell death is the introduction and/or generation of reactive species in the cellular environment by the application of cold physical plasma, resulting in the activation of redox signaling pathways. These latest developments encourage considering new treatment options. Of particular interest might be the possibility to promote the uptake of plasma-generated species by the combination with PEF-exposures. Hence, we review the latest developments in clinical and experimental ECT and PEF research in oncology and rationalize a combined treatment with plasmas for palliative therapy in patients.

1. Pulsed electric fields for tumor treatments

With a 5-year survival rate of only 20% [1], effective palliation is eventually often necessary for malignant melanoma patients. Electrochemotherapy (ECT) is frequently used in Europe, for the palliative treatment of cutaneous and subcutaneous malignant melanoma with relatively successful response rates of about 80% [2–4]. ECT proved to be more effective than conventional chemotherapy and often allows for avoiding surgery [5]. ECT uses pulsed electric fields (PEFs) with durations in the range of microseconds (μsPEF), and thus predominantly cellular plasma membranes are affected, affording permeabilization. Commonly used drugs in ECT are bleomycin and cisplatin. Generally, bleomycin is not taken up by cells, but it will accumulate intracellularly when cells are exposed to PEFs [6]. Once bleomycin enters the cell, it elicits DNA single- and double-strand breaks, leading to rapid cell death. Bleomycin is more toxic in fast-dividing tumor cells compared to quiescent non-malignant cells because unrepaired strand breaks mainly affect mitotic cells [7]. By facilitating bleomycin uptake only at the tumor site via ECT, the treatment achieves a high degree of selectivity, concomitantly alleviating systemic side effects [4]. Bleomycin can be administered intratumorally (i.t.) as well as intravenously (i.v.). The i.v. injection was found to be more effective especially in the treatment of large tumors [8].

Conversely, cisplatin is only injected i.t. [9]. Cisplatin diffuses through cell membranes also without PEFs, mediated by the copper transporter Ctr1 [10]. Inside the cell, cisplatin interferes with

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transcription and/or DNA replication mechanisms. Furthermore, it induces the receptor/extrinsic and the mitochondrial/intrinsic apoptosis pathways, as well as other signal transduction pathways, e.g., calcium signaling [11].

In addition to facilitating drug-uptake, ECT transiently modifies local vasculature and thus blood flow, which helps retaining drugs within the treated area [12,13]. Moreover, ECT-mediated damage of endothelial cells increases the antitumor efficiency due to the lack of vascularization, leading to nutrient depletion and hypoxia [14–17].

The first clinical study on ECT was conducted by Mir et al. with bleomycin in 1991, in which a clear antitumor effect was demonstrated for head and neck squamous carcinoma [18]. Afterward, a number of clinical studies provided more evidence for a therapeutic effect in cutaneous and subcutaneous metastasis of various tumor types, including malignant melanoma, basal cell carcinoma, and adenocarcinoma of the breast [2,19–21]. The original ESOPE (European Standard Operating Procedures of Electrochemotherapy) study was initiated in 2004 to define a standard operating procedure for ECT [9]. In 2018, these standard operating procedures were updated [22]. If the tumor size exceeds 3 cm or the number of nodules surpasses seven, general anesthesia and an intravenous drug injection should be considered. For the i.t. injection of bleomycin (concentration: 1000 IU/ml) or of cisplatin (concentration: 1 mg/ml), a dose of 0.25–1 ml/cm^3 is suggested depending on the tumor size. A dose of 15,000 IU/m^2 is recommended for the i.v. injection of bleomycin. The use of plate electrodes is advised for small and superficial tumor nodules, whereas needle electrodes are suitable for the treatment of thicker and deep-seated nodules. In the case of plate electrodes, eight electric pulses of 100-µs duration and field strength of 1.3 kV/cm are now the recommended procedure. Needle electrodes should be used in a similar fashion but only with an electric field of 1 kV/cm in amplitude. A frequency of 1 Hz or 5 kHz is advised, with higher frequencies helping to minimize muscle contractions during the procedure [5,9].

A more recent development is the introduction of genes instead of drugs into cells by PEFs (electrogenetherapy). Genes for protein targets with an immunomodulatory effect, such as interleukin (IL-12), are introduced locally via electrotransfer to induce systemic anticancer immune responses [23]. A respective proof-of-concept study was performed in canine mast cell tumors [24]. Replacing chemotherapeutic drugs with calcium is another novel strategy in PEF-assisted tumor treatment. Calcium overload causes cell death by necrosis and, thus, an immune stimulation, which was found to be as effective as traditional ECT [25]. Conceivable, ECT may, therefore, benefit also from the combination with cold physical plasma by facilitating the uptake of cytotoxic reactive species that are provided by plasma.

Without the combination of agents that are introduced into cells, e.g., chemotherapeutic drugs, µsPEFs have no relevant anticancer effect [26]. Conversely, exposures to pulsed electric fields with pulse durations shorter than the charging time of the cellular plasma membrane, i.e., in the nanosecond range (nsPEF), and sufficient field strengths, can interact with subcellular structures [27]. These nsPEFs effectively generate small pores (nanopores) in intracellular membranes but also in the outer cell membrane [28,29], induce apoptosis via several pathways [30], and mediate antivascular effects in vitro and in vivo [31–33]. Associated rapid cell death and short treatment times reduce the risks of therapy resistance and minimize side effects [34]. Exposures to nsPEFs have further been shown to inactivate cancer stem cells, which play a crucial role in tumor relapse [34]. Compounds that are generated by physical plasmas are presumably small enough to pass through pores that are created by nsPEF in the outer cell membrane. Plasma-exposures have been found to instigate in part similar processes, in particular, apoptosis. A combination with nsPEF might therefore potentially foster even synergistic effects.

2. Review of clinical experience with electrochemotherapy

The most extensive experience with exposures to pulsed electric fields for clinical therapies is based on the treatment of tumors by electrochemotherapy. Forty-nine case reports and studies were conducted between 1991 and 2018, investigating the efficacy of ECT [2,9,12,18–21,26,35–75]. Either bleomycin or cisplatin was administered in particular for malignant melanoma or breast cancer patients but also some other cancers, such as basal and squamous cell carcinomas, head and neck cancer, Kaposi's sarcoma, as well as non-surface tumors. In most of the studies, enrolled patients were treated for several lesions. Details on the investigations and results are summarized in Table 1. Two conceptually different electrode systems were used for the delivery of pulsed electric fields. In one approach, needle-configurations [9,12,20,21,38–40,44,46–62,64–66,68–75] were chosen, breaching the skin barrier and permitting PEF-delivery deep inside the tumor. However, needle-electrodes can be associated with some bleeding. The other approach relies on electrodes that are only in contact with the tumor without breaking the skin and hence applying PEF from the outside. Ideally, the entire tumor is exposed to the PEF if completely enclosed by the electrodes [2,9,18–21,26,35–43,45,48,51,52,55,56,59,62,66,69,72,73]. For intense exposures, i.e., a large number of pulses, as they are often used for tissue ablation by irreversible electroporation, or longer pulses, in particular in the millisecond range that are often used for gene transfer, tissue heating, and thermal damage, especially close to the electrodes, cannot be entirely avoided [76–79].

For the clinical ECT studies, exclusively exposures with pulse durations of 99 or 100 µs were applied. Field strengths of 0.9–1.3 kV/cm were investigated, although most studies relied on delivery with 1.3 kV/cm (for plate electrodes) or 1 kV/cm (for needle electrodes) according to the ESOPE recommendations. Likewise, mostly eight pulses were applied for PEF-exposures, but also four and six pulses were investigated. In most studies, conducted after the release of the ESOPE guidelines in 2006, a bleomycin concentration of 1500 IU/m^2 was used i.v. and a concentration of 250–1000 IU/cm^3 for intratumoral injections as recommended. The cisplatin concentration varied from 0.5–500 mg/cm^3 [9,26,42,45]; aside from one study using 20 mg/m^2 [43].

Notable is especially the success of ECT for sending malignant melanoma tumors into remission [2,9,12,19,20,26,38,40–44,46–49,51,63,73,75]. Of the sixteen studies that describe the results for individual tumors, twelve reported a complete tumor remission (CTR) for more than 50% of the treated lesions, with three of them finding a more than 90% success. Conversely, less than 20% showed no tumor response (NTR). In contrast, the response of patients is much lower, i.e., the overall complete patient's response (CPR) was around 40%, and more than 30% revealed no patient's response (NPR) [52,64,66,73].

Of the eight studies on basal cell carcinoma, 100% reported an overall response of the treated tumors [19,20,26,37,39,40,44,72]. Four of the studies even reported a CTR of more than 90% [26,39,40,72]. There are nine studies also investigating the response of squamous cell carcinomas [18,20,26,35,44,59,66,67,72]. The studies on the individual tumor response reported an overall response rate of at least 80%. Of the five studies evaluating the whole patient's response, one revealed a progressive disease (PD) for 80% of the patients [66] and the other four an overall response of at least 65% [59,67,69,74]. Four studies on Kaposi's sarcoma were also very promising [55,56,62,69]. An overall CPR over 75% could be achieved.

Thirteen studies investigated the response of breast cancer [20,21,36,44,45,49,51–54,57,61,64,66,75]. The overall response rate of individual tumors was at least 70%, with individual CTRs between 10–100%. Studies analyzing the whole patient's response exhibited NPR or PD up to 38%. The CPR varied from 8–63%.

Table 1
Clinical studies using ECT as a treatment for different cancer types, including melanoma, basal cell carcinoma, squamous cell carcinoma, and breast cancer. The studies are listed chronologically. The type of study, the number of patients enrolled, the treated cancer type, the treatment procedure, and the results are compiled.

Year	Type of study	Patients enrolled	Cancer type	Treatment procedure	Results of ECT treatment	Evaluation after ECT/ECT cycles	Ref.
1991	First clinical trial	7	HNSCC	Bleomycin: 10 mg/m^2 or 15 mg/m^2, intravenous injection PEFs: 4 or 8 × 100 µs pulse duration, field strength of 1.3 kV/cm or Electordes: stainless steel stripes	CR: 17 tumors (54.8%)[a] PR: 6 tumors (19.3%)[a] SD: 6 tumors (19.3%)[a] NR: 2 tumors (6.5%)[a]	6–48 d/2 (2 patients)	[18]
1993	Phase I/II Trial	8	HNSCC	Bleomycin: 10 mg/m^2, intravenous injection PEFs: 4 or 8 × 100 µs pulse duration, field strength of 1.3 kV/cm Electrodes: stainless steel stripes	CR: 23 tumors (57.5%)[b] PR: 6 tumors (15.0%)[b] NE: 11 tumors (27.5%)[b]	12–250 d/2 (2 patient); 3 (1 patient)	[35]
1995	First Clinical Experience	2	Melanoma	Bleomycin: 10 mg/m^2, intravenous injection PEFs: 8 × 100 µs pulse duration, field strength of 1.3 kV/cm Electrodes: two external electrodes	CR: 22 tumors (91.7%)[b] NR: 2 tumors (8.3%)[b]	3 months/2–3	[2]
1996	Extension of initial Phase I trial	7	HNSCC Breast Adenocarcinoma	Bleomycin: 10 or 15 mg/m^2, intravenous injection PEFs: 4 or 8 × 100 µs pulse duration, field strength of 1–1.3 kV/cm Electrodes: two stainless steel strips with round corners	CR: 1 tumor (10%)[b,c] PR: 1 tumor (10%)[b,c] SD: 5 tumors (50%)[b,c] PD: 2 tumors (20%)[b,c] NE: 1 tumor (10%)[b,c]	–/2 (1 patients); 3 (1 patient)	[36]
1996	Clinical study	2	Basal cell carcinoma	Bleomycin: 10 U/m^2, intravenous injection PEFs: 8 × 99 µs pulse duration, field strength of 1.3 kV/cm Electrodes: capilier electrodes	CR: 1 tumor (16.7%)[b] PR: 5 tumors (83.3%)[b]	12 weeks/–	[37]
1996	Observation	5	Melanoma	Bleomycin: 5 mU/mm^3, intralesional injection PEFs: 8 × 99 µs pulse duration; field strengths of 1.3 kV/cm Electrodes: plate or needle electrodes	CR: 18 tumors (78%)[a] PR: 4 tumors (17%)[a] NR: 1 tumor (5%)[a]	12 weeks/–	[38]
1996	Phase I/II Trial	6	Melanoma (3) Basal cell carcinoma (2) Adenocarcinoma (1)	Bleomycin: 10 u/m^2, intravenous injection (1–1.5 u/min) PEFs: 8 × 99 µs pulse duration, field strengths of 1.3 kV/cm Electrodes: plate electrodes	Melanoma: CR: 3 tumors (30%)[b*] PR: 2 tumors (20%)[b*] NR: 5 tumors (50%)[b*] Basal cell carcinoma: CR: 1 tumor (17%)[b*] PR: 5 tumor (83%)[b*] Adenocarcinoma: CR: 2 nodules (100%)[b*]	12 weeks/–	[19]
1997	Clinical study	20	Basal cell carcinoma	Bleomycin: 0.005–0.01 u/m^3, intratumoral injection PEFs: 99 µs pulse duration, field strength of 0.1 kV/cm Electrodes: caliper or needle array electrodes	CR: 53 tumors (98.1%)[b*] NE: 1 tumor (1.9%)[b*]	~18 months/2–3 (4 tumors)	[39]
1998	Clinical study	34	Basal cell carcinoma (20) Melanoma (12) Kaposi's sarcoma and Squamous cell carcinoma (2)	Bleomycin: 0.0008–0.0075 u/m^3, intralesional injection PEFs: 6 (needle array) or 8 (caliper) × 99 µs pulse duration, field strength of 1.3 kV/cm Electrodes: plate or needle array electrodes	Basal cell carcinoma: CR: 51 tumors (94.4%)[b*] PR: 3 tumors (5.6%)[b*] Melanoma: CR: 75 tumors (89.3%)[b*] PR: 8 tumors (9.5%)[b*] NR: 1 tumor (1.2%)[b*] Kaposi's sarcoma and squamous cell carcinoma: CR: 4 tumors (80%)[b*] PR: 1 tumor (20%)[b*]	12 weeks/–	[40]
1998	Independent Clinical Trials of 5 cancer centers	50	Basal cell carcinoma (10) Melanoma (20) Squamous cell carcinoma (17) Breast adenocarcinoma (2) Salivary gland adenocarcinoma (1)	Bleomycin: 18 u/m^2 or 27 u/m^2 (rapid bolus 30 s duration), intravenous injection; 10 u/m^2 (1.5 u/min), intravenous injection 0.25–1.0 u (30 s duration), intraturmoral injection PEFs: 4, 6 or 8× field strength 1.3 kV/cm Electrodes: needle array, caliper or two stainless steel strips electrodes	Basal cell carcinoma: CR: 24 tumors (75%)[b*] PR: 8 tumors (25%)[b*] Melanoma: CR: 75 tumors (52.8%)[b*] PR: 56 tumors (39.4%)[b*] NR: 11 tumors (7.8%)[b*] Squamous cell carcinoma: CR: 33 nodules (42.8%)[b*] PR: 15 nodules (19.5%)[b*] NR: 8 nodules (37.7%)[b*] Adenocarcinoma: CR: 22 tumors (100%)[b*]	6–27 months/ -	[20]
1998	Phase I/II Trial	4	Basal cell carcinoma (1) Melanoma (2) Squamous cell carcinoma (1)	Cisplatin: 0.009–0.5 mg/mm^3, intratumoral injection PEFs: 4 × 100 µs pulse duration, field strength of 1.3 kV/cm Electrodes: two parallel stainless steel electrodes	Melanoma: CR: 13 tumors (100%)[b] Basal cell carcinoma: CR: 4 tumors (100%)[b] Squamous cell carcinoma: CR: 1 tumor (50%)[b] CR: 1 tumor (50%)[b]for 9 months	7–11 months/3 (1 tumor) 8 (1 tumor)	[26]

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Table 1 (*continued*)

Year	Type of study	Patients enrolled	Cancer type	Treatment procedure	Results of ECT treatment	Evaluation after ECT/ECT cycles	Ref.
2000	Observation	1	Melanoma	Bleomycin: 0.0008–0.0075 U/m^3, intratumoral injection PEFs: 8 × 99 μs pulse duration; field strength of 1.2 kV/cm Electrodes: needle electrodes	CR: 9 tumors (100%)[a]	2 weeks/1	[12]
2000	Clinical study	4	Melanoma	Bleomycin: 10 mg/m^2, intravenous injection PEFs: 4 or 8 × 100 μs pulse duration, 1-1.3 kV/cm Electrodes: platinized metallic flat parallel electrodes	CR: 5 nodules (9.1%)[b*] PR: 46 nodules (83.6%)[b*] NR: 4 nodules (7.3%)[b*]	2 weeks – 2 months/2 (1 patient)	[41]
2000	Phase II Trial	10	Melanoma	Cisplatin: 0.01 mg/mm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1.3 kV/cm Electrodes. two parallel stainless steel electrodes	CR: 56 tumors (68%)[b] PR: 8 tumors (10%)[b] SD: 12 tumors (15%)[b] PD: 6 tumors (7%)[b]	4 weeks/NE	[42]
2000	Phase I/II Trial	9	Melanoma	Cisplatin: 20 mg/m^2 intratumoral injection PEFs: 8 × 100 μs pulse duration; field strength of 1.3 kV/cm Electrodes: two parallel stainless steel electrodes	CR: 3 tumors (11.1%)[b] PR: 10 tumors (37.0%)[b] NR: 11 tumors (40.7%)[b] PD: 3 tumors (11.1%)[b]	4 weeks/1	[43]
2001	Phase II Trial	15	Basal cell carcinoma (9) Melanoma (2) Squamous cell carcinoma (2) Breast cancer (2)	Bleomycin: 0.005–0.0075 u/m^3, intralesional injection PEFs: 100 μs pulse duration, field strength of 1.3 kV/cm Electrodes: needle array electrodes	Basal cell carcinoma: CR: 7 tumors (77.7%)[a] PR: 2 tumors (22.3%)[a] Melanoma: CR: 3 tumors (23.1%)[a] PR: 8 tumors (61.5%)[a] NR: 2 tumors (15.4%)[a] Squamous cell carcinoma: PR: 2 tumors (100%)[a] Breast cancer: CR: 8 tumors (58%)[a] PR: 6 tumors (42%)[a]	~8.6 months/1–3	[44]
2004	Clinical study	6	Breast cancer	Cisplatin: 0.01 mg/mm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1.3 kV/cm Electrodes: plate electrodes	CR: 4 tumors (33%)[b] PR: 8 tumors (66%)[b]	5–10 weeks/1	[45]
2005	Phase II Trial	19	Melanoma	Bleomycin: 1 U/cm^3, intratumoral injection PEFs: 100 μs pulse duration, field strength of 1.1 kV/cm Electrodes: needle electrodes	CR: 13 tumors (72%)[b] PR: 1 tumor (5%)[b] NR: 3 tumors (18%)[b] PD. 1 tumor (5%)[b]	12–24 weeks/1	[46]
2006	Clinical study	12	Melanoma	Bleomycin: 1 mg/cm^3, intralesional injection PEFs: 6 × 100 μs pulse duration, field strength of >0.6 kV/cm Electrodes: needle electrodes	CR: 17 tumors (56.7%)[b] PR: 3 tumors (10%)[b] NR: 1 tumors (3.3%)[b] PD: 3 tumors (10%)[b] NE: 6 tumors (20%)[b]	12 weeks/1	[47]
2006	ESOPE	61	Melanoma (32) Carcinoma (27) Sarcoma (2)	Bleomycin: 15,000 IU/m^2, intravenous injection 250–1000 IU/cm^3, intratumoral injection Cisplatin: 0.5–2 mg/cm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1.3 kV/cm (plate) 1 kV/cm (needle) Electrodes: plate or needle array electrodes	CR: 126 tumors (73.7%)[b, c] PR: 19 tumors (11.1%)[b, c] NR: 26 tumors (15.2%)[b, c] Melanoma: CR: 66.3%[b, d]	60–380 days/1	[9]
2008	Clinical study	14	Melanoma	Bleomycin: 15 mg/m^2, intravenous injection PEFs: 8 × 100 μs pulse duration, field strength of 1.3 kV/cm (plate) 1 kV/cm (needle) Electrodes: plate or needle electrodes	CR: 136 tumors (58%)[b] PR: 80 tumors (34%)[b] NR: 17 tumors (8%)[b]	8 weeks/1	[48]
2009	Clinical study	52	Melanoma Breast cancer Sarcoma Squamous cancer Head and neck cancer	Bleomycin: 15,000 IU/m^2, intravenous injection 250–1000 IU/cm^3, intratumoral injection PEFs: Information NE Electrodes: needle electrodes	CR: 125 tumors (47%)[e,c] PR: 126 tumors (47%)[e,c] NR: 16 tumors (6%)[e,c]	1 month/1	[49]
2011	Clinical Study	1	Liver cancer	Bleomycin: 15,000 U/m^2 (27.45 mg), intravenous injection PEFs: 8 × 100 μs pulse duration, field strength, of >0.9 kV/cm Electrodes: needle electrodes	CR: 1 patient (100%)[a,f]	16 months/1	[50]

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Table 1 (*continued*)

Year	Type of study	Patients enrolled	Cancer type	Treatment procedure	Results of ECT treatment	Evaluation after ECT/ECT cycles	Ref.
2011	Phase II Trial	52	Melanoma (21) Breast cancer (15) Adenocarcinoma (5) Basal cell carcinoma (5) Squamous cell carcinoma (3) Other (3)	Bleomycin: 8.5 mg/m^2, intravenous injection 0.25–1 ml/cm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1.3 kV/cm (caliper) 1 kV/cm (needle) Electrodes: plate or needle array electrodes	CR: 58 tumors (60%)[e,c] PR: 18 tumors (19%)[e,c] SD: 11 tumors (11%)[e,c] PD: 7 tumors (7%)[e,c] NE: 3 tumors (3%)[e,c]	> 60 days/2 (11 patients)	[51]
2011	First Clinical Trial in Greece	52	Melanoma (5) Other skin tumors (7) Head and neck tumors (14) Breast cancer (9) Other solid tumors (17)	Bleomycin: 15,000 IU/m^2, intravenousinjection 250–1000 IU/cm^3, intratumoral injection PEFs: as described in the ESOPE project Electrodes: plate or needle array electrodes	Melanoma: CR: 3 patients (60%)[b*] PR: 2 patients (40%)[b*] Other skin tumors: CR: 3 patients (42.9%)[b*] NR: 1 patient (14.3%)[b*] NA: 3 patients (42.9%)[b*] Head and neck: CR: 9 patients (64.3%)[b*] PR: 4 patients (28.6%)[b*] NR: 1 patient (7.1%)[b*] Breast cancer: CR: 2 patients (22.2%)[b*] PR: 6 patients (66.7%)[b*] NA: 1 patient (11.1%)[b*] Other solid tumors: CR: 13 patients (76.5%)[b*] PR: 3 patients (17.6%)[b*] NA: 1 patient (5.9%)[b*]	2 months/>1 (6 patients)	[52]
2012	Preliminary report	12	Breast cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: information NE Electrodes: needle electrodes	CR: 107 tumors (75.3%)[e] PR: 24 tumors (17%)[e] NR: 11 tumors (7.7%)[e]	30–354 days/1 (8 patients), 2 (4 patients)	[53]
2012	Phase II Trial	35	Breast cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8 × 100 μs pulse duration, field strength of 0.9-1 kV/cm Electrodes: needle electrodes	CR: 22 patients (62.8%)[e] PR: 8 patients (22.9%)[e] NR: 5 patients (14.3%)[e]	~32 months/up to 2	[54]
2012	Phase II Trial	23	Kaposi's sarcoma	Bleomycin: 15 mg/m^2, intravenous injection PEFs: as described in the ESOPE project Electrodes: needle or plate electrodes	CR: 12 patients(52.2%)[e] PR: 4 patients(17.4%)[e] PD: 5 patients(21.7%)[e] NA: 2 patients(8.7%)[e]	~1.5 years/2 (5 patients) 3 (2 patients)	[55]
2012	Clinical study	18	Kaposi's sarcoma	Bleomycin: 15 U.I., intravenous injection PEFs: as described in the ESOPE project Electrodes: as described in the ESOPE project	CR: 16 patients (88.9%)[b] PR: 2 patients (11.1%)[b]	4 weeks/2 (7 patients) 3 (2 patients)	[56]
2012	Phase II Trial	17	Breast cancer	Bleomycin: 15,000 IU/m^2, intravenous injection 1000 IU/cm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1 kV/cm Electrodes: needle electrodes	CR: 1 patient (8.3%)[e] PR: 1 patient (8.3%)[e] SD: 9 patients (75%)[e] PD: 1 patient (8.3%)[e]	48–94 days/2 (2 patients)	[57]
2012	Clinical Trial	15	Squamous cell carcinoma (13) Basaloid carcinoma (1) Merkel cell carcinoma (1)	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: information NE Electrodes: needle electrodes	CR: 19 tumors (61.5%)[e] PR: 10 tumors (32.5%)[e] SD: 1 tumor (3%)[e] PD: 1 tumor (3%)[e]	8 weeks/up to 3	[58]
2013	A preliminary study	8	Squamous cellular vulvar cancer	Bleomycin: 15 0000 UI/m^2, intravenous injection PEFs: as described in the ESOPE project Electrodes: as described in the ESOPE project	CR: 5 patients (62.5%)[e] PR: 1 patient (12.5%)[e] NR: 1 patient (12.5%)[e] PD: 1 patient (12.5%)[e]	1 month/1	[59]
2014	Phase II Trial	34	Soft-tissue sarcoma	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8 × 100 μs pulse duration, field strength of 0.91–1.00 kV/cm Electrodes: hexagonal-array, needle electrodes	CR: 13 patients (38.2%)[e] PR: 15 patients (44.2%)[e] SD: 3 patients (8.8%)[e] PD: 3 patients (8.8%)[e]	2 months/up to 4	[60]
2014	Clinical study	55	Breast cancer	Bleomycin: 15,000 IU/m^2, intravenous injection 250–1000 IU/cm^3, intratumoral injection PEFs: 8 × 100 μs pulse duration, field strength of 1 kV/cm Electrodes: needle array electrodes	CR: 22 patients (40%)[e] PR: 26 patients (47.3%)[e] SD: 7 patients (12.7%)[e]	2 months/~2	[61]
2014	Clinical Study	19	Kaposi's sarcoma	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: as described in the ESOPE project Electrodes: plate or needle electrodes	CR: 19 patients (100%)[e]	3–28 months/2 (3 patients) 3 (2 patients)	[62]

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Table 1 (*continued*)

Year	Type of study	Patients enrolled	Cancer type	Treatment procedure	Results of ECT treatment	Evaluation after ECT/ECT cycles	Ref.
2014	Clinical study	30	Melanoma	Bleomycin: information NE PEFs: information NE Electrodes: information NE	After first ECT: CR: 440 tumors (67.3%)[b] PR: 214 tumors (32.7%)[b] After second ETC: CR: 581 tumors (88.8%)[b] PR: 73 tumors (11.2%)[b]	4 weeks	[63]
2014	Clinical study	39	Melanoma (20) Breast cancer (7) Squamous cell carcinoma (5) Basal cell carcinoma (2) Angiosarcoma (2) Merkel cell carcinoma (1) Kaposi's cell carcinoma (1)	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8 × 100 µs pulse duration, field strength of 1 kV/cm Electrodes: hexagonal array, needle electrode	Melanoma: CR: 2 patients (10%)[e] PR: 9 patients (45%)[e] SD: 3 patients (15%)[e] PD: 6 patients (30%)[e] Other tumors: CR: 7 patients (36.8%)[e] PR: 8 patients (42.1%)[e] PD: 4 patients (21.1%)[e]	At least 6 months/1	[64]
2015	Retrospective cohort study	125	Breast cancer	Bleomycin: 15,000 IU/m^2, intravenous injection 250–1000 IU/cm^3, intratumoral injection PEFs: 8 × 100 µs pulse duration, field strength of 1.3 kV/cm (plate) 1 kV/cm (needle) Electrodes: plate or needle array electrodes	CR: 66 patients (58.4%)[e] PR: 36 patients (31.8%)[e] SD: 8 patients (7.1%)[e] PD: 2 patients (1.8%)[e] NA: 1 patient (0.9%)[e]	2 months/1	[21]
2015	Phase I/II Trial	13	Pancreatic cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8–96 × 100 µs pulse duration, field strength of 0.91–1.00 kV/cm Electrodes: hexagonal or linear needle electrodes	PD: 1 tumor (7.7%)[e] SD: 11 tumors (84.6%)[e] NE: 1 tumor (7.7%)[e]	~36 days/–	[65]
2015	Retrospective multicenter analysis	56	Melanoma (20) Breast cancer (13) Squamous cell carcinoma (15) Lymphoma/sarcoma (8)	Bleomycin: 15 mg/m^2, intravenous injection PEFs: 8 × 100 µs pulse duration, field strength of 1.3 kV/cm (plate) 1 kV/cm (needle) Electrodes: plate or needle electrodes	Melanoma: CR: 4 patients (20%)[e] PR: 6 patients (30%)[e] SD: 4 patient (20%)[e] PD: 6 patients (30%)[e] Breast cancer: CR: 1 patient (7.7%)[e] PR: 5 patients (38.5%)[e] SD: 2 patients (15.4%)[e] PD: 5 patients (38.5%)[e] Squamous cell carcinoma: CR: 1 patient (6.7%)[e] PR: 2 patients (13.3%)[e] PD: 12 patients (80%)[e] Lymphoma/sarcoma: PR: 6 patients (75%)[e] SD: 1 patient (12.5%)[e] PD: 1 patient (12.5%)[e]	–/~2	[66]
2015	Phase II Trial	25	Squamous cellular vulvar cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: as described in the ESOPE project Electordes: plate electrodes	CR: 13 patients (52%)[e] PR: 7 patients (28%)[e] SD: 3 patients (12%)[e] PD: 2 patients (8%)[e]	1 month/1	[67]
2016	Phase II Trial	29	Bone metastases	Bleomycin: 15 µ/m^2, intravenous injection PEFs: as described in the ESOPE project, 8 × field strength of 1 kV/cm Electrodes: needle electrodes	PR: 1 patient (3.4%)[e] SD: 17 patients (58.6%)[e] PD: 2 patients (6.9%)[e] DOD: 7 patients (24.1%)[e] NE: 2 patients (6.9%)[e]	3 months/–	[68]
2016	Phase II Trial	55	Non-melanoma head and neck skin cancer	Bleomycin: 15 mg/m^2, intravenous injection PEFs: as describes in the ESOPE project Electrodes: hexagonal or linear needle or plate electrodes	CR: 33 patients (60.0%)[e, c] PR: 17 patients (30.9%)[e, c] SD: 4 patients (7.3%)[e,c] PD: 1 patient (1.8%)[e,c] Basal cell carcinoma: CR: 16 patients (67%)[e,d] Squamous cell carcinoma: CR: 13 patients (52%)[e,d] Merkel cell carcinoma: CR: 2 patients (100%)[e,d] Angiosarcoma: CR: 1 patient (100%)[e,d] Kaposi's sarcoma: CR: 1 patient (100%)[e,d]	~8 months/1 (32 patients), 2 (11 patients), 3 (11 patients)	[69]

(*continued on next page*)

Table 1 (*continued*)

Year	Type of study	Patients enrolled	Cancer type	Treatment procedure	Results of ECT treatment	Evaluation after ECT/ECT cycles	Ref.
2017	Pilot study	5	Colorectal liver metastasis	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 96 × 100 µs pulse duration, field strength of 0.73 kV/cm Electrodes: linear or hexagonal needle electrodes	CR: 3 tumors (33.3%)[e] SD: 1 tumor (11.1%)[e] PD: 5 tumors (55.5%)[e]	6 months/–	[70]
2017	Phase I/II Trial	19	Locally advanced pancreatic cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8–96 × 100 µs pulse duration, field strength of 0.4–1.0 kV/cm and 0.91–1.0 kV/cm Electrodes: linear, hexagonal, or variable needle electrodes	RECIST: SD: 16 patients (84.2%) PD: 2 patient (10.5%) NE: 1 patient (5.3%) Choi response: PR: 18 patients (94.7%) NE: 1 patient (5.3%) PERCIST: PR: 6 patients (31.6%) SD: 3 patients (15.8%) PD 1 patient (5.3%) NE: 9 patients (47.4%)	1 month/–	Data from [71]
2017	Clinical study	12	Basal cell carcinoma (42 tumors) Squamous cell carcinoma (10 tumors)	Bleomycin: 10,000 IU/m^2, intravenous injection 15,000 IU/m^2, intravenous injection PEFs: information NE Electrodes: plate or needle electrodes	Basal cell carcinoma: CR: 41 tumors (97.6%)[e] PR: 1 tumor (2.4%)[e] Squamous cell carcinoma: CR: 10 tumors (100%)[e]	2 months/1	[72]
2017	Prospective cohort study	151	Melanoma	Bleomycin; 1000 IU/m^2, intratumoral injection 15,000 IU/m^2, intravenous injection PEFs: as describes in the ESOPE project, 8 × 100 µs Electrodes: plate or needle array electrodes	Target lesions: CR: 229 tumors (58.1%)[e] PR: 77 tumors (19.5%)[e] SD: 79 tumors (20.0%)[e] PD: 6 tumors (1.5%)[e] NE: 3 tumors (0.8%)[e] 114 patients matching criteria: CR: 55 patients (48.2%)[e] PR: 29 patients (25.4%)[e] SD: 26 patients (22.8%)[e] PD: 3 patients (2.6%)[e] NE: 1 patient (0.9%)[e]	60 days/1 (123 patients), 2 (21 patients), 3 (5 patients), 4 (2 patients)	[73]
2017	EURECA project	43	Mucosal head and neck cancer	Bleomycin: 15,000 IU/m^2, intravenous injection PEFs: 8 × 100 µs pulse duration, field strength of 1 kV/cm Electrodes: finger, hexagonal, or linear electrodes	CR: 8 patients (18.6%)[e] PR: 16 patients (37.2%)[e] SD: 10 patients (23.2%)[e] PD: 3 patients (7.0%)[e] NE: 6 patients (14.0%)[e] Squamous cell carcinoma: OR: 22 patients (65%) Adenoid cystic carcinoma: OR: 2 patients (67%)	8 weeks/1	[74]
2018	Phase II Trial	7	Melanoma (1) Breast cancer (6)	Bleomycin: 500 and 1000 IU/cm^3, intratumoral injection Calcium chloride: 4.5 and 9 mg/cm^3, intratumoral injection PEFs: 8 × 100 µs pulse duration, field strength of 0.4 kV/cm Electrodes: linear array needle electrodes	Bleomycin: CR: 13 tumors (68.4%)[e,c] PR: 3 tumors (15.8%)[e,c] PD: 3 tumors (15.8%)[e,c] Calcium: CR: 12 tumors (66.7%)[e, c] PR: 1 tumor (5.5%)[e,c] SD: 3 tumors (16.7%)[e, c] PD: 2 tumors (11.1%)[e,c]	6 months/1	[75]

CR: complete response; PR: partial response; NR: no response; NE: not evaluable; SD: Stable disease; PD: progressive disease; OR: objected response; DOD: dead of disease; HSNCC: head and neck squamous cell carcinoma.

[a] Ambiguous response criteria.
[b] WHO response criteria.
b* Similar to WHO response criteria.
[c] Response of all tumor types.
[d] Only the CR/OR was evaluated.
[e] Response Evaluation Criteria in Solid Tumors (RECIST).
[f] Metastasis were removed in surgery.

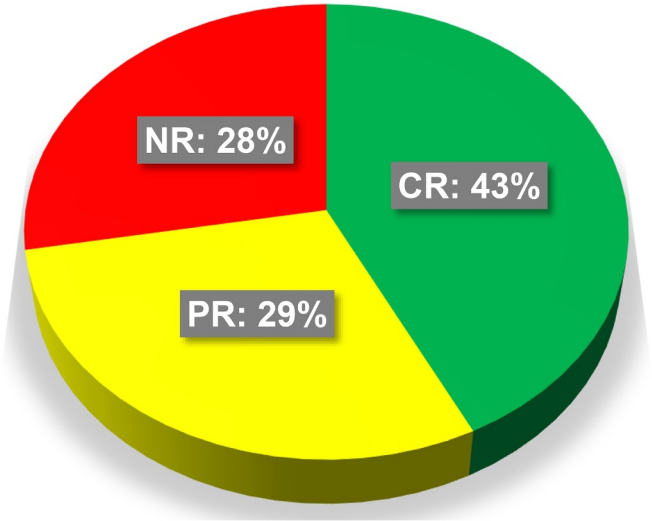

Fig. 1. Mean total ECT response rates of patients evaluated by RECIST in studies summarized in this work. Three of ten cancer patients in the palliative stage do not benefit from ECT. CR: complete response; PR: partial response; NR: no response.

A newer development is the application of ECT to non-surface tumors, such as liver and pancreatic cancer, as well as bone metastasis. The results for pancreatic cancer seem to be promising for a palliative purpose. A stable disease (SD) was achieved in over 80% of the tumors or patients [65,71]. The one study analyzing the response of bone metastasis revealed at least over 60% SD or partial patient's remission (PPR) [68]. The results were more ambiguous for the ECT treatment of liver cancer. One study achieved a CPR of 100% [50], whereas another study revealed 55.5% PD [70].

The comparison of overall responses of different studies, as presented in Table 1, was difficult, due to the use of different response criteria. However, in the latest studies, and in most that were focusing on the patient's response, the Response Evaluation Criteria in Solid Tumors (RECIST) was applied. The overall responses of these studies, which analyzed the whole patient's response by RECIST, are summarized in Fig. 1.

The ESOPE study concluded that best tumor control was achieved with bleomycin i.v., followed by cisplatin i.t. and bleomycin i.t. [9]. However, average patient response rates encourage active research on ECT to enhance its efficacy, e.g., by focusing on nanosecond pulses and other combination approaches, to identify additional therapeutic potentials.

3. Response to pulsed electric fields at a molecular level

ECT is based on the application of relatively long, i.e.in the range of microseconds, and moderate PEFs, i.e., in the order of several hundred V/cm, in combination with chemotherapeutic drugs. Accordingly, the cell membrane integrity is transiently compromised, allowing cytostatic drugs to enter the cell [4,80,81]. Regardless, cells are instead damaged by biochemical responses that are achieved by the drug than by PEF-exposure. Conversely, nsPEF-exposures were shown to affect cells more directly. Nanopores are created throughout the outer cell membrane, as well as subcellular membranes that allow small molecules and ions to pass. However, the inherent potential and considerable interest in nsPEFs for cancer treatment is that no chemotherapeutics are needed to induce cell death. Instead, nsPEFs can reliably induce different apoptotic pathways that are triggered as a result or in conjunction with subcellular membrane permeabilization events [82–86].

In the general model for electroporation (the formation of pores by exposures to pulsed electric fields), a water defect within the phospholipid bilayer is introduced first [87]. During subsequent pore construction, the phospholipid head groups are reorganized in each leaflet around the defect. Next, during pore maturation, additional water and head groups migrate into the pore-forming a channel for molecular transport. After the removal of the electric field, the pore destabilizes. Water and head groups migrate out of the pore, leading to a decrease in pore size. In the subsequent pore deconstruction, the head groups separate. The pore dissolution is finished when water and cytoplasm are separated again by the phospholipid barrier [87,88]. Interestingly, the sensitivity of the membrane towards electropermeabilization can be enhanced via oxidation [89].

In addition to the outer cell membrane and intracellular membranes, nsPEFs can also affect the cytoskeleton. One 60-ns pulse with 15 kV/cm applied to HeLa and Jurkat cells, was shown to cause a breakdown of the cytoskeleton [90]. Although HeLa cells detach from the surface, recovered cells could adhere again, forming new colonies. In contrast, Jurkat cells, which are suspension cells, did not recover. In the same study, nsPEFs were also found to compromise the telomere/nuclear envelope structure [90]. The cytoskeleton degradation was also shown in B16F10 cells in which caspase activation and cytoskeleton breakdown occurred simultaneously [84]. Subcellular effects are not exclusive to nsPEF but generally more pronounced than for pulses of longer durations. An effect on the cytoskeleton was also shown using μsPEF. In human umbilical vein endothelial cells (HUVECs), a disruption of microfilament and microtubule cytoskeletal networks was observed after three electric pulses of 4–32 kV/cm, a frequency of 1 Hz, and a duration of 100 μs [91].

One well-studied effect of nsPEFs is the increase of the intracellular calcium level [85,92,93]. In HL-60 cells, treated with a single pulse of 60 ns and 15 kV/cm, the increase of intracellular calcium is similar, regarding magnitude and kinetics, as after treatment with the purinergic agonist UTP. It was also shown that calcium is mobilized from the endoplasmic reticulum, and subsequent calcium influx is initiated through store-operated calcium channels or minimum size pores in the cytoplasmic membrane [29,94]. The dissipation of mitochondria membrane potential was described to be calcium-dependent in N1-S1 HCC cells [85]. Most of the calcium-regulated events are mediated by proteins; hence, it was suggested that nsPEFs activate the mitochondrial permeability transition pore (mPTP) complex in the mitochondrial membrane [93]. Indeed, nsPEFs were demonstrated to non-thermally inactivate the cAMP-dependent protein kinase which is the prototype of the protein kinase superfamily [93]. Therefore, nsPEFs were proved to be able to directly affect proteins. Nevertheless, an involvement of mPTP in the nsPEF-induced dissipation of the mitochondrial membrane potential was not found [93].

Exposures to nsPEFs induce different cell death mechanisms, depending on the cell line and field strength. In Jurkat and HL-60 cells, treated with pulses of 60 ns and 60 kV/cm, cytochrome c release and caspase activation were observed, suggesting a caspase- and mitochondria-mediated signaling pathway [95]. However, in B16F10 cells, treated with ten 300-ns pulses and a field strength up to 60 kV/cm, no cytochrome c release was observed, suggesting an extrinsic apoptotic pathway without mitochondrial amplification [84]. In the same study, caspase activation was found to be independent of calcium. In HCT116p53$^{+/+}$ and HCT116p53$^{-/-}$ cells, treated with 300-ns-PEFs of 60 kV/cm, caspase activation was induced prior to cytochrome c release, also indicating mitochondria-independent mechanisms [96]. Furthermore, in E4 squamous carcinoma cells, treated with ten pulses of 300 ns and field strengths up to 60 kV/cm, nsPEFs triggered various cell death mechanisms. Bid cleavage was found to be partly caspase- (55–60%) and calcium-dependent (40–45%) whereby extracellular calcium was predominately responsible, suggesting a primarily extrinsic pathway [97]. It should be mentioned that excessive nsPEFs-exposures (e.g., large pulse numbers with high electric fields) could also induce necrosis which was, for example, shown for U937 cells treated with 600 pulses of 10 ns and 100 kV/cm [98].

The effectiveness of nsPEFs for cancer treatment was shown in various animal experiments [31,99–101]. Notably, Nuccitelli et al. were able to eliminate all melanomas in four- to six-week-old female Nu/Nu mice (immunodeficient, hairless, albino) after only one treatment. For this, they used newly developed suction electrodes with optimized parameters of 2000 pulses, 100 ns in duration, a field strength of 30 kV/cm, and a frequency of 5–7 pulses/s. They could also demonstrate that the untreated surrounding healthy tissue was not damaged [100].

The first-in-human trial using nsPEF for cancer therapy was performed in 2014. Three patients with a total of ten basal cell carcinoma lesions were treated using 100–1000 electric pulses with a duration of 100 ns and a field strength of 30 kV/cm that were applied at 2 pulses/sec. Seven of the lesions underwent complete regression and exhibited no scars after healing [102].

4. Responses to cold physical plasma at a molecular level

Cold physical plasmas are partially ionized gasses that can be generated in many different ways [103]. The prime hallmark of plasmas that are used for biomedical applications is an ion temperature close to body temperature, allowing their application to cells and tissue without thermal harm [104]. Plasmas generate UV photons, electric fields, electrons and ions, mild thermal radiation, and visible light as well as reactive oxygen and nitrogen species (ROS/RNS)[105,106]. The latter have been identified to be of crucial relevance in mediating plasma effects for cells in vitro [107–109]. Technical limitations in identifying reactive species directly in tissues have hampered the proof of their prime importance in tissue responses, although this is suggested by evidence of oxidative events, e.g., for plasma-treated skin [110,111]. In addition, plasma components other than ROS/RNS, above all electromagnetic fields, may contribute to biological responses, following plasma-exposures [112–114].

Plasma devices have already entered the market for medical procedures in dermatology [115]. In patients, plasma treatments support the healing of chronic ulcers [116–119], decrease microbial burden in wounds and on skin [120–122], increase tissue oxygenation [123–125], and enhance epidermal drug absorption [126]. The atmospheric pressure plasma jet application on the skin is generally considered safe [127]. Yet, early and late skin damages as a consequence of helium plasma treatment were suggested in an in vivo mouse study [128]. The side effects depended on the treatment time, the gas flow, and probably the gas source. Another mouse study revealed no apparent side effects of argon plasma treatment after a one-year follow-up risk assessment [129].

An important target of cold plasmas is the cell membrane. Plasma-derived reactive species cause lipid peroxidation and changes in membrane-bound proteins (e.g. ion channel proteins) [130–132]. Following results from redox biology, this affects signaling pathways in the cell's interior [133] although a causative relationship between membrane oxidation products and subsequent cellular signaling has not been formally established . Some reactive species have also the potential to penetrate the cell membrane directly, e.g. H_2O_2 via aquaporin channels and superoxide anions through the chloride channel-3 [134]. In principle, plasma treatment follows hormetic dose-response curves [135], with often stimulatory effects for low and toxic effects for high treatment intensities. Extensive plasma-exposures can trigger necrosis [136]. As for in vitro studies, many observations could be assigned to plasma-derived redox-active species and several biologicals effects of cold atmospheric plasma can be confirmed by findings from the field of redox biology [107,134,137]. However, the composition and dynamic of reactive species created by plasma is very complex and can therefore not easily be substituted by other means. It is important to note that plasma-derived ROS deliver biological responses depending on their concentration. These hormetic effects – low concentrations have a different, sometimes opposite effect compared to high concentrations of a given substance [135] – explain why at low concentrations/plasma treatment times, beneficial effects are observed e.g. in wound healing [138], while the potential for the treatment of cancer lies at higher concentrations/plasma treatment [139].

Plasma-exposures are highly toxic for malignant cancer cells [140–142] which has been shown to be of relevance in several animal models [143–145], including melanoma [136,146,147]. In vitro, plasma treatment mediates a wide range of cellular responses in tumor cells: apoptosis, growth inhibition, cell cycle arrest, as well as cytoskeletal and mitochondrial damage [148–150]. (Similar responses were also observed for different PEF-exposure regimens as discussed above.) Moreover, the observed decrease of cell migration is mediated through the loss of adhesion capability. It was shown for human melanoma cells that plasma not only compromises the cell morphology but also reduces the activity of adhesion proteins, such as integrins and focal adhesion kinase [151,152]. Both play an important role in survival, growth, and metastasis formation of melanoma cells [153–155].

The exact mechanism of plasma-induced apoptosis is still under debate. In A549 cells, anti-apoptotic Bcl-2 mRNA was significantly down-regulated after indirect plasma treatment. Bcl-2 is the inhibitor of pro-apoptotic Bax whose mRNA level remained unchanged. Both are involved in the mitochondrial/intrinsic pathway. Conversely, caspase 3/7 activity was not elevated after the treatment, suggesting a caspase-independent mitochondrial pathway [156]. In contrast, Jurkat cells directly treated with a dielectric barrier discharge revealed an increased Bax, Bcl-2, p53, and caspase-8 expression after 48 h leaving the Bax/Bcl-2 ratio unchanged. Nevertheless, the pro-apoptotic function can also be mediated by Bcl-2-independent mechanisms. Thus, the contribution of the mitochondrial/intrinsic pathway still has to be further investigated. The involvement of receptor/extrinsic pathway is strongly indicated by the significant increase of caspase-8 and p53 up-regulation [157]. Plasma-derived ROS also have the potential to affect cell membranes, including mitochondria [144,158,159], promoting mitochondrial dysfunction, and mitochondrial-mediated apoptosis (intrinsic pathway). Yet, the exact biochemical pathways of plasma-derived ROS have not been fully elucidated. It is known that some ROS, such as ozone [160] and nitric oxide [161], can penetrate through membranes, while others, such as hydrogen peroxide, can be transported into cells via aquaporins [162]. At the same time, diffusion of other, i.e. charged species, such as peroxynitrite [163] and the superoxide anion [164], across eukaryotic cell membranes is very limited. Hence, plasma treatment of biological targets likely yields several reactions in the extracellular and intracellular compartment. Which species are needed at what concentrations to achieve a desired biological outcome is still subject of current investigations.

5. Combining pulsed electric fields and cold plasma – potential benefits

The comparison of effects of PEFs and cold plasma on mammalian cells shows that on the one hand some similar mechanisms can be addressed while on the other hand different mechanisms, which are induced, could benefit from each other, e.g. for the induction of apoptosis. In general, effects that were observed for PEF with either microsecond or nanosecond duration are not mutual exclusive. Therefore, for the moment no definite conclusion can be drawn on a respective 'better' choice for the combination with cold plasma. The same holds true for ideal sequences of such combination treatments. From the above-mentioned studies, it can be reasoned that potential additive or synergistic responses, especially of μsPEFs and plasma, are most likely achieved via respective effects on cell membranes. This was already suggested by studies on the combination of plasma and μsPEF for bacterial inactivation [165]. For mammalian cells, it was previously also shown that oxidized membranes are more sensitive to electroporation [89]. Adding to this, Breton and Mir claimed that long-term

effects of μsPEF-exposure, which they define as electropermeabilization, are caused by lipid peroxidation of cell membranes [166]. Lipid peroxidation is also observed after plasma treatment [158]. It might therefore be reasonable that plasma treatment could potentially enhance a subsequent μsPEF-application, leading to stronger effects, for example lower transmembrane voltage thresholds and/or increased pore radii and/or pore distributions. In principle, this might be more important for cellular plasma membranes, separating the interior of the cell from the cellular environment, than for intracellular membranes, segmenting for instance the endoplasmic reticulum, the mitochondria, and the nucleus. The simple reason is that plasma-derived reactive species are in most cases short-lived and have limited diffusion distances [167]. If plasma generated species reach the cell membrane, i.e. if they are not scavenged before interacting, species will quickly react with intracellular proteins. Lipids are readily decreasing the immediate oxidative action of short-lived species prior to intracellular membrane contact [168]. Our experimental evidence supports the view of plasma-derived reactive species acting on the cell membranes but not directly on mitochondria [169,170], which differs from the effects observed for nsPEF [30]. Hence, for species-rich plasma, combined with low intensity nsPEFs or UV radiation, additive effects are reasonable. Potential additive effects on intracellular membranes such as mitochondria are likely a product of secondary reactions within cells [170]. Conversely, μsPEF- or nsPEF-treatment prior to plasma application could facilitate the permeation of plasma-derived ROS/RNS due to electropermeabilization. This might lead to a direct increase of reactive species inside the cell, resulting in oxidation of biomolecules [171]. In particular, nsPEF-exposures of tissues have been predicted to permit a comprehensive tissue permeabilization [172]. With respect to practical applications, this might allow reaching also deeper skin layers by plasma generated species which is so far elusive for topical treatments with cold plasma. It is noteworthy that also plasma treatment alone can lead to non-lethal cell membrane poration, allowing for intracellular accumulation, e.g. of tumor-toxic nanoparticles [173], dextran molecules of up to 6.5 nm diameter [174], or plasmids [175]. Hence, a third strategy could be combining plasma and PEF technology into one device.

In addition to consequences on cellular membranes and associated responses, other molecular mechanisms can likewise potentially be enhanced by a combination of PEFs and cold plasma. In particular, the intracellular calcium increase caused by nsPEFs and the enhanced intracellular ROS concentration after plasma treatment might result in mutually enhanced mechanisms. Cytochrome c is normally bound to cardiolipin at the inner mitochondrial membrane. The dissociation of cytochrome c and cardiolipin is the first important step of cytochrome c release and thereby of the mitochondrial apoptosis pathway. This can be stimulated by ROS or calcium which binds to cardiolipin. Furthermore, ROS can mediate mitochondrial dysfunction via lipid peroxidation [176].

Plasma and nsPEFs are also both known to activate the receptor/extrinsic apoptosis pathway [84,96,97,157]. Apoptosis that is induced by nsPEF does not necessarily rely on calcium increase or mitochondria depolarization [84,96,97]. Likewise, plasma was shown to upregulate p53 and caspase-8 [157]. This suggests that at least an additive effect could be accomplished by a combination treatment with specific experimental setups and parameters.

All treatments, μsPEF, nsPEF, and plasma, are also able to affect the cytoskeleton and cell adherence characteristics, at least to some degree [84,90,91,151]. The detachment from the surrounding extracellular matrix can lead to anoikis, a form of programmed cell death of anchorage-dependent cells [151]. This effect can already be observed under relatively low intensities of both nsPEF- and plasma-exposure. However, the clinical relevance of these mechanisms remains to be established.

6. Clinical perspective of combining ECT and physical plasma treatments

With a non-responder rate of about 10–30% in patients suffering metastatic melanoma and breast cancer (Fig. 1), there is room for improvement in clinical palliation with ECT (Fig. 2) [9,48]. Cold physical plasma has shown promising tumor-toxic results in animal models [177–179] as well as in patients [139,180,181]. Its potential importance in the treatment of malignant melanoma was reviewed recently [182]. An obvious strategy of combining both physical treatment would be either a preconditioning of metastatic tumors with plasma treatment, i.e. to improve the permeability of cell membranes, followed by ECT, or a post-treatment following ECT, i.e. complementing or even

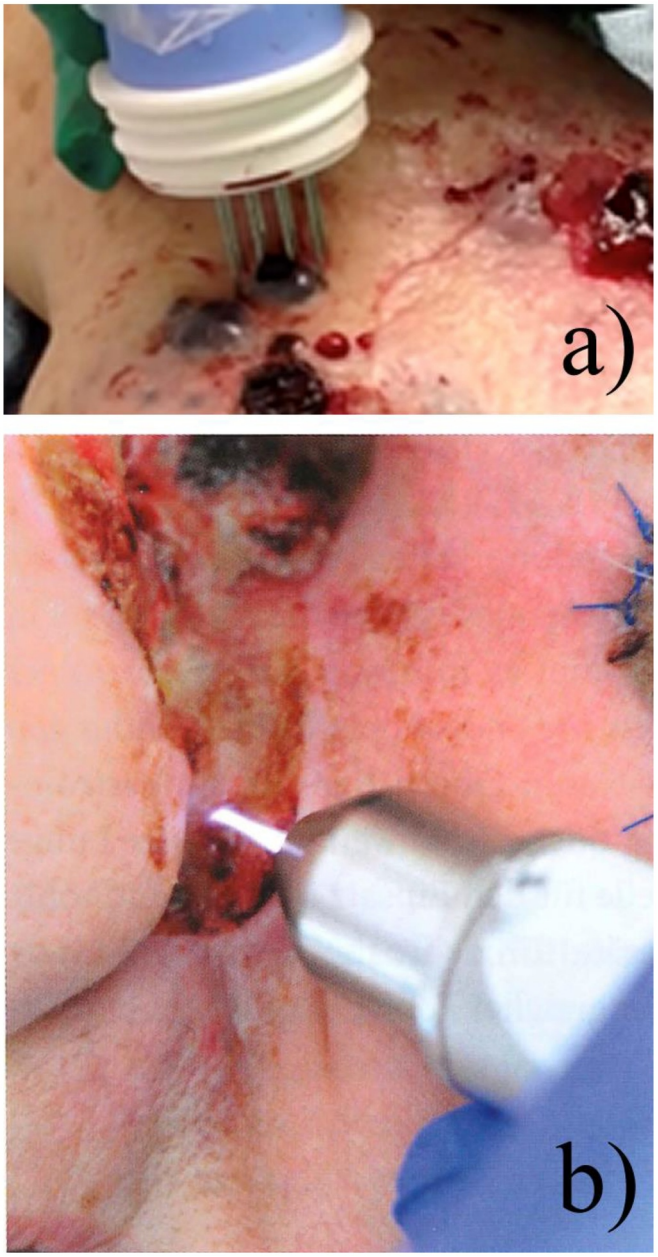

Fig. 2. (a) ECT-treatment of a tumor patient in palliative care suffering from end-stage metastatic melanoma. (b) Plasma-treatment of an infected malignant tumor to reduce microorganism load and to stimulate tumor regression [193].

supplementing cytostatic drugs. It is worth mentioning that promising results were already achieved in a first test, combining the use of plasma and ECT in a melanoma mouse model [183].

From a clinical perspective, there are still open questions and technical challenges regarding a possible combination of ECT and plasma treatment. In non-ulcerating metastatic lesions, the tumors are located beneath the stratum corneum of the skin. This thick layer of the upper epidermis scavenges the majority of plasma-derived reactive species and other effectors. Our own unpublished observational studies of treatments with the kINPen® MED (neoplas tools GmbH, Greifswald, Germany) [184], in patients suffering from cutaneous and subcutaneous melanoma metastasis, confirmed a lack of effect by direct plasma treatments, also after multiple treatments of the same intradermal tumor site. Therefore, the question is how μsPEF-exposure used in ECT could enhance the accessibility of subcutaneous tumor tissue to plasma-derived reactive species. Perhaps the most obvious option would be the use of the channels pinched into the tissue created from the μsPEF device. Yet, bleeding caused by needle electrodes impedes externally provided reactive species to reach the tumor site. This, and the hemostatic activity of physical plasma [185–190], limits the access of plasma-derived reactive species to cancer cells. Conversely, at least species that are forming in the fluid can potentially be delivered or provided even more easily. A more elegant strategy would take advantage of the increased cell permeability, up to the stratum corneum, introduced with the PEF-exposure, concomitant with or followed by a topical plasma application. However, whether this enhances diffusion rates of plasma-derived reactive species into deeper layers of the skin remains to be established.

In ulcerating tumors, where some of the tumor cells are directly accessible to topical treatment procedures, the situation is different. In these cases, plasma treatment could achieve additional effects, either by pre-conditioning the tumor lesions with plasma to ECT or by providing an additional attack to tumor tissue following ECT. However, by far not all metastatic lesions in metastatic melanoma patients are ulcerating [191].

With these limitations in mind, what is the technical requirement for combining both physical technologies in metastatic cancer palliation in non-ulcerating tumors? Ideally, the plasma generation would be integrated into the μsPEF delivery device that is used for ECT. This would allow for treatment with plasma either before and/or after electroporation. Most plasma-derived ROS are short-lived (nanoseconds to seconds) and quickly interact with biomolecules in close vicinity. If plasma-derived ROS were to predispose cancer cells for ECT treatment, or if ECT-treatments would facilitate the entry of plasma-derived ROS, both physical modalities need to be applied sequentially in strictly short time frames in order to maximize the effect of ROS. Such an approach may not only be technically challenging but difficult from a regulatory affair's point of view. The application of high voltage pulses carries the risk of electrical discharges within or on top of treated tissue [192] and only a limited number of electrical discharges would be permissible. This also applies to nsPEFs, which – in contrast to μsPEFs – confer direct tumor-toxic effects, but are not accredited for palliative procedures in cancer patients, yet. However, the potential benefits of combining both methods warrant the effort of respective investigations.

7. Conclusion

The success of ECT demonstrates the potential for the combination of pulsed electric field exposures with therapeutic drug deliveries in cancer treatment. Exposure to medical plasmas is a rather novel approach for the generation of therapeutically active compounds. Membrane effects of PEFs and of the ROS/RNS generated by plasma might mutually reinforce each other as well as the various cell death mechanisms induced by plasma and nsPEF. Combining both methods can therefore provide a possibility for a more effective treatment of tumors in comparison with the individual techniques alone.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Ethical statement

None.

Financial disclosure

None.

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