UNIVERSITÀ
DEGLI STUDI
DI PADOVA

DOTTORATO DI RICERCA IN

ONCOLOGIA E ONCOLOGIA CHIRURGICA

XXV ciclo

Sviluppo delle applicazioni cliniche dell'elettroporazione nel trattamento delle metastasi cutanee e dei tumori dei tessuti molli

Dottorando: Dott. Luca Giovanni Campana

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Anno Accademico 2011-2012

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Development of clinical electroporation for the treatment of skin metastases and soft tissue tumors

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RIASSUNTO

Introduzione. Il principio fisico dell'elettroporazione (EP) cellulare (la temporanea permeabilizzazione della membrana citoplasmatica per mezzo di campi elettrici) è stato applicato al trattamento dei tumori con l'intento di aumentare la concentrazione dei farmaci antiblastici (bleomicina e cisplatino) all'interno della cellula tumorale e, di conseguenza, la loro azione citotossica. Fino al 2006, anno di standardizzazione della procedura dell'elettrochemioterapia (ECT), l'esperienza clinica con questo tipo di trattamento era limitata ad alcuni studi di efficacia.

Scopo. Lo scopo del progetto è stato quello di indagare l'efficacia dell'ECT in popolazioni omogenee di pazienti oncologici (pazienti con metastasi cutanee da melanoma, recidiva da carcinoma mammario sulla parete toracica, recidiva di sarcoma dei tessuti molli, tumori del distretto capo-collo). Inoltre, è stata indagata la fattibilità e l'efficacia di una nuova apparecchiatura, in grado di applicare il trattamento anche a tumori profondi. Infine, è stata indagata, a livello clinico e preclinico la possibilità di migliorare l'efficacia del trattamento tramite, rispettivamente, la valutazione degli effetti immunologici del trattamento (induzione dei Toll-like receptors, TLRs) e la valutazione dell'effetto sensibilizzante della butionina sulfossimina (BSO) all'azione della bleomicina.

Metodi. Complessivamente, sono stati disegnati quattro studi clinici prospettici di fase II, di cui tre conclusi ed uno attualmente in corso di arruolamento, ed uno retrospettivo. Inoltre, sono stati analizzati i campioni tessutali di alcuni pazienti prima e dopo il trattamento per analizzare i livelli di espressione dei TLRs. In fine, sono stati condotti degli studi su linee cellulari per valutare l'effetto sensibilizzante della BSO al trattamento con BLM + EP.

Risultati. In tutti gli istotipi tumorali trattati l'ECT ha dimostrato, a livello locale, un livello di attività superiore a quello dei regimi di chemioterapia attualmente impiegati. Questo si è tradotto in un apprezzabile controllo locale dei tumori trattati, a fronte di una tossicità limitata e perlopiù locale.
Nei campioni tissutali prelevati prima e dopo il trattamento, la variazione dei livelli dei TLRs non è risultata significativa, ma un elevato infiltrato linfocitario nella lesione è risultato correlato con il grado di risposta locale. In vitro, il pretrattamento con BSO aumenta la citotossicità del trattamento con BLM + EP. Un risultato ancillare è stato anche il riscontro di un' aumento della tossicità del melphalan, un farmaco attualmente impiegato nella terapia loco regionale del melanoma, in associazione all'EP. La applicazione clinica dell'ECT ha permesso di rilevare (anche grazie alla collaborazione multidisciplinare) alcuni aspetti tecnici (applicazione e gestione del campo elettrico sui tessuti) meritevoli di essere migliorati per aumentare l'efficacia del trattamento ed il numero di pazienti che possono beneficiarne.

Conclusioni. L'ECT si è dimostrata un trattamento dotato di un'elevata attività nel trattamento delle recidive di melanoma, carcinoma mammario, sarcomi dei tessuti molli e tumori del distretto capo-collo. L'esperienza clinica iniziale con una nuova apparecchiatura ed elettrodi dedicati ha rilevato la possibilità di trattare efficacemente anche i tumori profondi. Il trattamento di ECT sembra non ha innalzato significativamente i livelli di TLRs nei tumori dei pazienti trattati, ma livelli elevati di infiltrato linfocitario nel tumore sono risultati correlati con livelli maggiori di risposta locale. La BSO è in grado di sensibilizzare in vitro i tumori al trattamento combinato con BLM ed EP. L'EP merita di essere ulteriormente indagata, anche in associazione al melphalan nei pazienti con melanoma.
In conclusione, l'ECT ha la potenzialità di essere implementata in campo oncologico attraverso la collaborazione multidisciplinare di oncologo, chirurgo, radioterapista, biologo ed ingegnere.

ABSTRACT

Introduction. The principle of cell electroporation (EP) (the temporary permeabilization of the cytoplasmatic membrane by means of electric fields) has been applied to the treatment of tumors with the intent of increasing the concentration of anticancer drugs (cisplatin and bleomycin) within the tumor cell and hence their cytotoxic effect. Until 2006, the year of standardization of the electrochemotherapy (ECT) procedure, clinical experience was limited to small heterogeneous series, mainly focused on local activity data in patients with superficial metastases.

Aims. The aim of this project was to investigate the efficacy of ECT in homogeneous populations of cancer patients of different histotypes (patients with skin metastases from malignant melanoma, recurrent breast cancer on the chest wall, recurrent soft tissue sarcomas and tumors of the head and neck area). Furthermore, it was investigated the feasibility and efficacy of a new device (electric pulse generator and dedicated needle electrodes), capable of applying homogeneous electric fields also to deep tumors. Finally, we investigated, both at clinical and preclinical level, the possibility of improving the effectiveness of ECT by, respectively, the evaluation of some immune effect of treatment (i.e., the induction of the Toll-like receptors, TLRs) and the evaluation of the tumor sensitizing action to ECT of buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis.

Methods. Overall, four prospective, phase-II, clinical trials were designed, three of them have been concluded and a fourth is still ongoing and patients' enrollment is open. Moreover, a retrospective study was performed on head and neck cancer patients. In some patients, tissue samples were analyzed before and after ECT, to evaluate the expression of TLRs. Finally, some in vitro tests were conducted on tumor cell lines to assess the sensitizing effect of BSO pre-treatment to BLM + EP administration.

Results. All the histotypes showed high local response rates and ECT activity was more pronounced than standard chemotherapy regimens employed in current oncology practice. Local response translated in an appreciable local control of the treated tumors, while toxicity was limited and mainly local.

TLRs levels in post-ECT tumor biopsies was not significantly different, compared with pre-ECT samples. However, the immune reaction seems to play a role since a high lymphocytic infiltrate into the electroporated lesions was associated with higher response rates. In vitro tests, BSO pretreatment enhanced the cytotoxicity of BLM + EP. Of note, we also found an increased toxicity of melphalan, a drug currently used in the treatment of locoregional melanoma, in association with EPs. Thanks to the multi-disciplinary collaboration and clinical case management, we have individuated some technical aspects that deserve further improvement in order to increase the effectiveness of treatment application and the number of patients who can benefit from it.

Conclusions. ECT has proved a highly active treatment in recurrent melanoma, breast cancer, soft tissue sarcomas and cancers of the head and neck region. The preliminary clinical experience with a new device and dedicated electrodes indicates the feasibility to treat percutaneously even large, deep-seated tumors. Although ECT treatment do not seem to raise the levels of TLRs in the electroporated tumors, however the levels of lymphocytic infiltration were associated with better local response. In vitro, BSO pre-treatment is able to sensitize tumor cells to the combined treatment of BLM and EP.

Electroporation deserves further investigation also in combination with melphalan, which is currently used in locoregional chemotherapy of in transit metastases from melanoma.

In conclusion, ECT has the potential to be implemented in the oncology field through the multidisciplinary collaboration of oncologists, surgeons, radiation oncologists, biologists and engineers.

INTRODUCTION

ELECTROCHEMOTHERAPY (ECT): BASIC CONCEPTS

The direct injection of therapeutic molecules into cells and tissues can be considered as the simplest method for drug delivery. However, the main drawback of this method is the low efficiency of drug bio-distribution and uptake. For 10 years now, the development of tumor-targeting and drug delivery methods in cancer treatment has become a major bio-medical research field in order to increase therapeutic benefits and reduce the side effects of therapeutic molecules. The common aspect of these methods is to deliver drugs into cells via plasma membrane modifications. Drug-loaded chemical vectors fuse with the plasma membrane or are endocytosed by the cell. Physical methods transiently destabilize the plasma membrane creating leaky structures (e.g. membrane defects or pores). Among these methods, electropermeabilization (also named electroporation, EP) has received increasing attention, particularly as a promising method for drug delivery [1]. In the past 25 years, electropermeabilization has proved to be safe and efficient for the delivery of nucleic acids (e.g. plasmid DNA, siRNA, antisense RNA), proteins (e.g. antibody) and antitumor drugs (e.g. cisplatin [CDDP], bleomycin [BLM]) across the plasma membrane [2-4]. Electropermeabilization-based cancer treatment methods are currently undergoing intensive exploration in the field of drug delivery [5], gene therapy [6], genetic vaccination [7] and irreversible electroporation [8]. The first medical application in the treatment of cancer is electrochemotherapy (ECT), which became a clinically approved treatment approach for cutaneous and subcutaneous tumors [9–11]. ECT is defined as a local treatment, which, thanks to cell membrane permeabilizing electric pulses, potentiates the cytotoxicity of non- or poorly permeant anti-tumor drugs [5].

Physical principles

Electroporation is a method of cell membrane permeabilization that is today widely used in biotechnology and medicine for delivery of drugs and genes into living cells [12]. It is alternative method for water sterilization and food preservation [13], and it is a prerequisite for cell electrofusion. The phenomenon of electroporation can be described as a dramatic increase in membrane permeability caused by externally applied short and intense electric pulses. Various theoretical models were developed to describe electroporation, among which the transient aqueous pore model is the most widely accepted. According to this model, hydrophilic pores are formed in the lipid bilayer of a cell membrane when it is exposed to external electric pulses. In the cell membrane, hydrophobic pores are formed by spontaneous thermal fluctuations of membrane lipids. In a cell exposed to an external electric field, the presence of an induced transmembrane potential provides the free energy necessary for structural rearrangements of membrane phospholipids and thus enables hydrophilic pore formation. Hydrophilic pores form only in a small fraction of the membrane exposed to electric field. Even though some attempts to visualize the changes in the membrane structure caused by electric pulse application were made [14], the structural reorganization and creation of hydrophilic pores has so far not been directly observed. All the data available until now have been obtained as an indirect evidence of membrane permeabilization, such as measurements of conductivity changes caused by electric pulse application and observations of molecular transport through the cell membrane. Cell membrane electroporation takes place because the cell membrane amplifies the applied external electric field, as its conductivity is several orders of magnitude lower than the conductivities of extra cellular medium and cell cytoplasm. The theoretical description of the transmembrane potential induced on a spherical cell exposed to electric field is known as Schwan's equation [15]. The induced transmembrane potential for a spherical cell can be calculated as:

$$UTI = -1.5rE\cos\varphi$$

where r is the radius of the cell, E is the strength of applied electric field, and φ is the angle between the direction of the electric field and the selected point on

the cell surface. The induced transmembrane potential and therefore maximum electroporation occur at the poles of the cell exposed to the electric field facing the electrodes (Fig. 1). Electroporation can be either reversible or irreversible, depending on parameters of the electric pulses. It is a threshold phenomenon: the induced trans membrane voltage imposed by external electric field should reach a critical value to trigger formation of transient aqueous pores in the cell membrane. The threshold membrane potential that needs to be reached in the cell membrane is between 200 mV and 1 V. For reversibility of electroporation, the membrane potential has to be kept below the critical value. In such conditions, the cell membrane recovers after electric pulse application. On the contrary, when the critical value is exceeded, irreversible electroporation takes place, resulting in cell membrane disintegration and loss of cell viability. The electroporation process consists of different phases. The first of them is pore formation, which is the cell membrane's response to the induced threshold membrane potential, and lasts a few microseconds. The second phase is a time dependent expansion of the pore size taking place in a time range of hundreds of microseconds to milliseconds, and lasts throughout the duration of pulses. The last phase is membrane recovery, which takes place after electric pulse application and consists of pore resealing, and lasts several minutes This resealing phase is strongly affected by temperature and cytoskeleton integrity [16-17]. The first phase of electroporation can be measured by changes in membrane conductivity and is related to short-lived transient pore formation, which does not contribute to molecular transport [18]. Molecular transport across the permeabilized cell membrane associated with electroporation is observed from the pore formation phase until membrane resealing is completed [19]. Electroporated membranes are also a prerequisite for associated membrane

phenomena termed electrofusion. During electric pulse application and immediately after it, the cell membrane is capable of fusion: it is in a so-called fusogenic state. In brief, electroporation is a useful technique in biotechnology and medicine for introduction of different molecules into the cell, electrofusion,

or water sterilization and food preservation. Among different theoretical models that describe electroporation, the transient aqueous pore model is most widely accepted. This model predicts hydrophilic pore formation as a response to induced external electric field on the cell membrane. Electroporation can be reversible or irreversible, depending on the electric pulse parameters used.

Electroporation is affected on the one hand by parameters of electric pulses and chemical composition of the media used and on the other by the characteristics of the cell that is exposed to the electric field. The effect of the electric pulse parameters and electroporation media are briefly described. The most important electric pulse parameters are *amplitude*, *duration*, *number*, and *repetition frequency*. If those parameters exceed the optimal values, irreversible electroporation takes place due to cell membrane disintegration and DNA damage, resulting in cell lysis. The choice of electric pulse parameters thus depends on the desired application. Some applications require reversible, while others require irreversible electroporation. For loading of foreign molecules into the cell, reversible electroporation is required. The choice of electric pulse parameters depends on the type of the foreign molecule that is being introduced. For small molecules, such as different drugs or fluorescence dyes, a train of relatively *short pulses* (time duration in range of microseconds to milliseconds) is sufficient. For large molecules, such as DNA, *longer pulses* (range of few milliseconds) or a combination of high-voltage short-duration pulses and low-voltage long-duration pulses is used. Besides the before mentioned parameters of electric pulses, different pulse shapes can also be used. The most frequently used are *exponential* and *square wave pulses*. One should be careful when comparing results obtained by different pulse shapes, as the membrane polarization process that takes place during the pulse application is different. Electric pulses can be applied in one direction or their orientations can be changed during the pulse application. Such protocols were successfully used for
electrochemotherapy and gene electrotransfer.

For introduction of small molecules, short electric pulses in a range of tens to hundreds of microseconds are generally used. The most important parameter is pulse amplitude. It should reach a threshold value at which the electroporation of cell membrane is triggered. Above the threshold value the increase in electroporation is obtained with increase of pulse duration and number of pulses (Fig. 2). The increase in pulse duration increases the electroporation of cells until a plateau is reached and further increase in number of pulses or its duration does not affect cell electroporation. At the same time the increase in pulse number and pulse duration affects cell viability. The following explanation for the relationship between the pulse amplitude and the pulse number or duration was proposed: increasing the pulse amplitude results in larger area of membrane electroporation with smaller extent of electroporation, while increase in pulse number or duration does not affect the electroporated membrane area but increases the extent of electroporation (Fig. 3).

Nevertheless, when increasing the duration of the pulse, one should also consider that longer pulses cause significant Joule heating of the sample. Systematic study of electric pulse parameters revealed that electroporation and cell viability are not related to the total electrical energy delivered. Further examinations of different parameters of electric pulses indicate complex dependence between electric pulse parameters and degree of electroporated cell membrane [20]. Another electric pulse parameter affecting electroporation of the cell membrane is pulse repetition frequency. When pulses are applied with high repetition frequency, above 1 kHz, the pause between two consecutive pulses is too short and does not allow cell membrane to return to pre-pulse state. From the experimental results it can be concluded that cell viability and cell membrane electroporation is optimal in the frequency range from 0.5 to 10 Hz and decreases at higher frequencies. For reversibility of electroporation, the membrane potential has to be kept below the critical value. In such conditions, the cell membrane recovers after the electric pulse application. On the contrary,

when critical value is exceeded, irreversible electroporation takes place, resulting in cell membrane disintegration and loss of cell viability.

The optimal conditions for introduction of macromolecules are different from optimal conditions for introduction of small molecules. Most experiments were performed with long, 5 to 10 ms pulses with relatively low pulse amplitude. When those results were compared with results obtained with higher voltage microsecond pulses, typically used for introduction of small molecules, it was established that many different pulse parameters are capable of delivering plasmid DNA into the cell. Protocols employing millisecond pulses are more efficient than microsecond pulses for long-term gene expression in vivo. The efficiency of gene electrotransfer into mammalian cells was first related to the pulse shape used, and exponentially decaying pulses were reported as more effective that the square wave pulses. Later, the use of combination of high-voltage and low-voltage pulses was suggested. High-voltage pulse causes electroporation of cell membrane, while the low-voltage pulse helps highly charged DNA entrance into the cell interior. A low-voltage pulse thus provides electrophoretic movement of DNA into the cell in *in vitro* conditions, or it can be a powerful driving force for improving interstitial transport of DNA during gene delivery *in vivo*. The effect of electrophoretic pulses was successfully used and demonstrated in *in vivo* experiments in mammalian tissues [21].
Nevertheless, the role of electrophoretic force in DNA movement across permeabilized membrane is questioned for in vitro gene electrotransfer as no contribution of electrophoretic force could be detected. Lately the effect of electrophoretic movement of DNA by low-voltage pulse has also been questioned for in vivo applications [22]. The effect of low-voltage electric pulse on the highly charged DNA is alternatively attributed to electrophoretic accumulation of DNA on the cell membrane. It has also been demonstrated by visualization of DNA interaction with the cell membrane that the electric field orientation plays an important role in gene electrotransfer. Similar to small molecules, asymmetric DNA uptake is observed during electroporation.

Nevertheless, DNA, unlike small molecules that enter cell cytoplasm on the membrane-facing cathode, enters the cell on the surface-facing anode. Another main difference between introduction of small molecules and DNA is that for successful gene electrotransfer, DNA has to be present in the medium before electric pulses are applied (Fig. 4) and the transport of the DNA through cell membrane takes place minutes after the pulse application. The complex between the DNA and the membrane forms only when the membrane is electroporated. If DNA is added after pulse application, no transfection can be observed. It was, however, demonstrated that transfection is successful if the DNA is added after the high-voltage pulse and before low-voltage pulse, but the level of DNA expression is lower.

Single-cell electroporation is a suitable tool for the study of basic electroporation mechanisms. A few attempts were made to observe ultra-structural changes related to electroporation; however, the process is too fast. Besides, chemical composition and fluid characteristics of the thin cell membrane make direct observation of primary membrane changes related to electroporation very difficult. The attempt was made to use rapid freezing scanning microscopy to determine the changes in membrane structure [14]; however, the size of the pores observed was 20 nm up to 120 nm, too large compared to theoretically estimated 1 nm, and the observed pores were most probably secondary structures. At the cell membrane level, the induced transmembrane potential was imaged by fluorescence probes sensitive to transmembrane potential changes induced by an external electric field. Temporal and spatial induction of transmembrane potential on the cell membrane that responds to externally applied electric field was observed with potentiometric dyes. The results obtained in those experiments on a single spherical cells are in good agreement with the theoretically calculated values obtained by Schwan's equation. The value of induced trans membrane potential sustainable for living cell electroporation was determined to be 1 V. Later the value of the induced trans membrane potential that triggers electroporation was

determined to be in the range of 200–500 mV. These values obtained by fluorescence imaging and calculations were further confirmed by direct measurement at the single-cell level using patch clamp technique.

The value of induced transmembrane voltage depends on the cell size, shape, and the position of the cell with respect to the direction of applied electric field. For a spheroidal cell, the maximum induced transmembrane potential strongly depends on its orientation with the respect to the electric field (Fig. 5). It is maximum when the spheroidal cell is parallel to the applied electric field. The distribution of induced transmembrane potential is asymmetric due to native transmembrane potential that is present in live cells. As the induced trans membrane potential caused by externally applied electric pulses is superimposed to the resting membrane potential of the cell, the side of the cell facing the anode is hyperpolarized while the side facing the cathode is depolarized The membrane labeling with fluorescent probes allows imaging of the membrane area affected by applied electric pulse. It was found that the membrane resting potential has a significant effect on asymmetric electroporation, especially when the induced trans membrane potential is close to the threshold voltage that triggers electroporation. This, however, is the case in majority of the applications in which cell viability needs to be preserved. The cell shape affects the site of cell membrane electroporation, and it is especially important in attached cells, as they are not at regular shape. The calculation of induced transmembrane potential on single cells, therefore, depends on the realistic cell shape that needs to be taken into account as it affects the calculated distribution of the induced transmembrane potential.

Although a single-cell model is a valuable tool for the study of basic mechanisms of electropotaion, it is not the best method to predict electroporation behavior in a tissue. As a tissue is composed of cells that are close to each other, dense cell suspensions represent an intermediate level between the single-cell level and the tissue. Neighboring cells, even if they are not in direct contact, affect each other due to mutual electrical shading [23].

For electroporation of cell suspensions, the proportion of the cells in the total volume is important. When they represent less than 1% of the volume fraction they behave as single cell, while for volume fraction greater than 10% or for clusters of cells, the induced transmembrane potential is affected by the suspension density. The fraction of electroporated cells decreases with increase in cell density and the resealing of cells in dense cell suspensions is slower. In dense cell suspensions, cell clusters, and multicellular spheroids it was found that the molecular tran sport is slower due to slower diffusion of molecules into the interior of such cluster or spheroid. Dense cell suspensions can serve as a model for tissues with homogeneous structure composed of similar cells in close contact; nevertheless, most tissues are not homogeneous.

Tissues are composed of different cell types that are irregularly shaped, are vascularized, and present different electrical properties. All the mentioned factors affect the distribution of electric field within the tissue and consequently its electroporation efficiency [24]. Furthermore, cells in tissue are connected by gap junctions for intracellular communications and transport, which change the electroporation behavior of such cells, and they behave as a single larger cell. For efficient tissue electroporation *in vivo*, the electric field distribution, which depends on electrode geometry, position, and electrical properties of the sample, is crucial. The electrical properties of biological tissue such as conductivity and permitivity change once the tissue is permeabilized and the electric field distribution is changed. The largest part of these changes is attributed to increased membrane conductivity due to electroporation. Changes in membrane conductivity need to be taken into account when performing electroporation with multiple needle electrodes and can be used for detection of cell membrane electroporation and for pulse delivery control. Recently these changes were used for regulating the output voltage for *in vivo* gene transfection. One of the major problems with respect to conductivity measurements *in vivo* is the inhomogeneous distribution of current density and electric field due to inhomogeneous and anisotropic properties of the tissue. For

successful tissue electroporation, anatomically based mathematical models are important tools for prediction of the outcome of the treatment [24].

Single-cell electroporation is a suitable tool for study of basic electroporation mechanisms. The situation is more complex in tissues as they are composed of cells that are in close contact with each other and their proximity affect electroporation. Besides, most tissues are not homogenous structures, they are composed of different cell types that are irregularly shaped, are vascularized, and have different electrical properties that affect current density and electric field distribution, all of these affecting electroporation effectiveness. Mathematical models are thus a valuable tool for predicting electroporation behavior of the tissue.

Pharmacological principles

Because of their physico-chemical properties (e.g. charge, molecular weight, hydrophilicity) and/or their deficiency of membrane transport mechanisms, most anti-tumor drugs are poor or non-permeant molecules and their anti-tumor efectiveness is limited. Several chemotherapeutic drugs have been tested on cancer cell lines for potential application in combination with electropermeabilization. The main ECT mechanism is electropermeabilization of tumor cells, which enhances drug cytotoxicity by facilitating significant drug uptake to reach intracellular targets (e.g. nuclear DNA, phospholipids) **(**Fig. 6). Thus, cell electropermeabi-lization increases cytotoxicity of some chemotherapeutic drugs from approximately 1.1 up to several thousand-fold. Nevertheless, tp date only two of these drugs that are currently clinically used in combination or not with other drugs have been recognized as potential candidates for ECT of cancers in clinical human and veterinari treatments: cisplatin (CDDP) and bleomycin (BLM). Cisplatin transport through the plasma membrane is also limited. 50% of cisplatin is conveyed through the plasma membrane by passive diffusion and the remainder is transported by receptors. Cisplatin has been most extensively characterized as a nuclear DNA damaging agent and the cytotoxicity of cisplatin has generally attributed to the

ability to form inter- and intra-strand DNA crosslinks **(**Fig. 7). This mechanism is believed to be the main cause of the anti-proliferative effect of cisplatin. In fact, only approximately 1% of intracellular platinum is bound to nuclear DNA. The great majority of the intracellular drug is available to interact with other nucleophilic sites on other molecules. Thus, cisplatin binds to mitochondrial DNA, induces endoplasmic stress, interacts with phospholipids and phosphatidylserine in membranes, disrupts the cytoskeleton and affects the polymerization of actin. Membrane electropermeabilization enables increased cellular flux and accumulation of the drug, which results in an enhancement of cisplatin cytotoxicity by up to 80-fold. The transport of bleomycin across the plasma membrane is achieved by receptors that internalize it via the endocytosis process. The low number of receptors exposed at the cell surface limits this process. Thus, this pathway limits the uptake of bleomycin and its cytotoxicity. The controlled exposure of cancer cells to electric pulses leads to increased membrane permeability and enables the direct access of bleomycin to the cell's cytoplasm. Bleomycin acts by inducing DNA strand breaks by an oxygen- and metal ion-dependent process. Indeed, bleomycin chelates metal ions, producing a pseudo-enzyme that reacts with oxygen to produce superoxide and hydroxide free radicals that cleave nuclear DNA. Moreover, these complexes also mediate lipid peroxidation and oxidation of other endogenous molecules such as proteins (Fig. 8). A few hundred molecules are only required for the cytotoxicity. Previous investigations showed that electropermeabilization of cancer cells potentiates the cytotoxicity of bleomycin up to several thousand-fold [25].

Antitumor action

Besides membrane permeabilization, Sersa and colleagues showed that the application of electric pulses to targeted tissues induces a transient, reversible and profound reduction of tumor blood flow (i.e. 80% decrease) [26–27]. This phenomenon, called *vascular lock*, allows drug entrapment in the targeted tissue up to several hours providing more time for the drug to act. The time

course of blood flow changes has been described. It follows the same two-phase pattern. The first rapid and short-lived vasoconstriction phase is followed by a much longer-lived second phase resulting from disrupted cytoskeletal structures and a compromised barrier function of microvascular endothelium causing endothelial

cell permeabilization and drug extravasation enhancement. The uptake of chemotherapeutic drugs into tumor vascular endothelial cells leads to unrepairable damages to tumor vessels and to a further decrease in tumor blood flow within hours after electrical treatment. Consequently, the ECT potentiates the destruction of tumor vasculature and breaks down the nutrient supply (e.g. oxygen, growth factors), which induces the cell death of tumor cells. In all this is the direct effect of ECT. Vascular disrupting contributes to the increase of antitumor effectiveness of bleomycin and cisplatin. Indeed, the anti-vascular effects of ECT are also exploited for palliative treatment of bleeding melanoma metastases, with immediate stop of bleeding and very good anti-tumor effectiveness. This anti-vascular effect could play a major role into the treatment of deep-seated vascular tumors.

In the 90s, Mir and colleagues first showed that the *host immune response* is essential for obtaining cures after ECT [28]. The tumor growth delay in immunocompetent mice was approximately twice than in immunodeficient mice. Thus, 80% of tumors cures were achieved in immunocompetent mice but none in immunodeficient mice [44]. On the basis of experimental fact, systemic ECTinduced immunity can be upregulated by additional treatment with immune-boosting molecules such as interleukin-2 and -12, and TNF-α. The antitumor effectiveness of such combinations was tested in different types of primary tumors, distantly growing tumors and induced metastases. Indeed, intratumoral IL-12 electrogene therapy has been proved to be very effective in local tumor control, having also a systemic effect. Moreover, intramuscular and peritumoral IL-12 electrogene therapy had also a pronounced systemic effect and when combined with other treatment (e.g. electrochemotherapy) resulted in

tumor cures [29]. The anti-tumor effectiveness of such molecules is due to the induction of adaptive immunity and innate resistance and anti-angiogenic action.

Equipment

Electroporation is used for different purposes and depending on the application one should chose the right electrodes to obtain the desired result. For different applications, different types of electrodes are available and can be classified according to their geometry into different groups: plate, needle, wire, and tweezers electrodes [30]. In certain cases, special electrodes are needed; for example, for individual-cell electroporation, specially designed microelectrodes are required. For treatment of large volumes of sample and for flow electroporation, electroporation chambers that allow efficient treatment were designed and successfully tested. They were successfully used for gene transfection or water treatment. The choice of most suitable electrodes for a given application depends also on the characteristics of the treated sample.

For reversible electroporation used in medicine, electrode design have to allow efficient electroporation and at the same time cause as little cell damage of the surrounding tissue as possible. In *in vivo* electroporation, electrical properties of the treated tissue have to be taken into account, as they vary significantly among different tissues. In electroporation, mathematical models taking into account the tissue conductivity changes can be very useful for proper electrode selection and their positioning with respect to the tissue that needs to be electroporated, since the electric field distribution can be efficiently modified by electrode geometry and their position during the pulse application. Irreversible electroporation, used in water sterilization and food preservation, where large volumes need to be treated and high electric fields need to be applied, requires different methodologies. For flow electroporation, it is crucial that the pulse delivery frequency is linked to the flow rate in such a way that each cell that passes electroporation chamber receives electric pulse treatment.

Liquid flow during electroporation affects causes cell elongation therefore electric field orientation with respect to cell is important. The choice of proper electrode shape and their position during the pulse application is crucial for successful treatment, as they affect the electric field distribution. The most appropriate electrode type and positioning depends on the application.

Pros and cons of ECT treatment

Although highly effective, up to now ECT has been limited to the treatment of superficial (cutaneous and subcutaneous) or low deep tumors (indicatively, 3 cm). Consequently, further developments are focusing on the treatment of internal tumors by the development of flexible electrodes, endoluminal electrodes, long needle electrodes and small-size electrodes.

Electrochemotherapy is currently used for the treatment of cutaneous and subcutaneous tumour nodules of any type of malignancies. The advantages for electrochemotherapy can be summarised as follows.

First, the effectiveness in tumour nodules of different histologies. Melanoma was the predominant tumour type in the first clinical trials, however, there are several reports demonstrating effectiveness of ECT on other types of recurrent tumours or metastases, like breast carcinoma, head and neck tumours, squamous cell carcinoma, basal cell carcinoma and others in more sporadic reports. The objective response rate of non-melanoma tumours was the same as of melanoma tumours 81%, which indicates on equal effectiveness of ECT on different tumour types. The rationale for the obtained results is simple, either bleomycin or cisplatin, when reaching intracellular targets, exert their cytotoxic action, if sufficient amount of the drug is present in the cells. Since electric pulses induce electropermeabilisation of the cells, in electrochemotherapy more drug is able to reach its intracellular targets,

the cell DNA, which explains the higher efficacy of these drugs in association with application of electric pulses to the tumours.

Second, the minimal side effects. Electrochemotherapy is easy and quick (i.e., 25 min) to perform, in majority of cases on out-patient basis (as clearly stated in the SOP). Therefore, it has minimal burden to the patients, since in most case its effectiveness was demonstrated after a single treatment. However, it could be repeated with equal antitumour effectiveness, if the tumour nodules recur or if new tumour nodules emerge. After treatment no specific care or dressing of the treated nodules is required. All these aspects, and the fact that electrochemotherapy can be performer also in patients with contraindications for surgical treatment or radiation therapy and in elderly patients, provide evidence that electrochemotherapy has substantial impact on quality of life in cancer patients with progressive disease. Furthermore, electrochemotherapy is performer with low doses of bleomycin or cisplatin therefore no sistemi side effects were observed.

Third, ECT iso f simple application. It be performed in general or local anaesthesia. In either of the procedures it is a simple procedure that can be, in the case of local anaesthesia, performer on an out patient basis. No extra technical skills are needed to perform the treatment, an 1 day training session is sufficient to perform the treatment according to the prepared SOP. Therefore, it is a procedure that can be performed also in developing countries and small hospitals, where other standard treatments are not readily available. In comparison with the complexity of other local or regional treatments like radiotherapy, isolated extremity perfusion and infusion it is much more simple.

Fouth, the possibilità of repetitive treatments. Electrochemotherapy is an effective treatment when sufficient drug concentration is obtained in the tumour nodules and the whole tumour volume is adequately covered by application of electric pulses, so that most of the tumour cells are electroporated. In the case of bigger tumour nodules that are not covered by electric field in single run of electric pulse application, several applications of electric pulses are required. In such cases viable tumour cells may remain, therefore recurrent or remaining tumour mass must be retreated. Electrochemotherapy is very effective in repetitive treatments as demonstrated in several clinical cases. The treatment

can be repeated in 3–6 weeks interval with the same treat-ment effectiveness as in the previous treatment.

Finally, the effectiveness in tumours emerging in pre-treated areas. ECT was so far tested in patients with progressive disease, where other standard treatment procedures have failed or were exhausted. In some cases the recurrent tumour nodules were in previously irradiated areas or in the area of the surgical field of the previously removed nodules. Clinical data demonstrated effectiveness in most of these cases, regardless of being in previously irradiated areas or in previously resected areas, or in the skin flap. In such cases standard interventions are no longer possible and electrochemotherapy provides treatment of choice for these tumours.

Indications to ECT

In medicine, electroporation is used in ECT in clinical practice for improved drug delivery for cancer treatment, and in preclinical trails for gene electrotransfer. From the point of view of medical applications, it is more convenient to use a high-repetition pulse frequency rather than 1 Hz pulse repetition, which is currently used in clinical trials. This is important when larger tumor nodules need to be treated and when multiple needle electrodes are used. In that case, a large number of pulses need to be delivered to each of the pairs of the electrodes, which would represent an unpleasant and a relatively long treatment time, if pulses were delivered at 1 Hz repetition frequency. The application of pulses with higher repetition frequency does not significantly affect the electrochemotherapy efficiency and the treatment is less unpleasant than application of pulses with standard 1 Hz repetition frequency. At the in vivo level, tissue vascular lock is observed due to disruption of blood vessel network after the application of high-voltage pulses. Consequently, the tissue oxygenation level is reduced by electroporation resulting in enhanced tumor cell death.

CLINICAL ELECTROPORATION: THERAPEUTIC APPROACHES

Electro-gene-deliery (EGT)

The goal of any gene transfer method is to deliver genes to desired cells in tissues and organs in an efficient manner with minimal toxicity. The main problem associated with nonviral gene transfer has been modest transgene expression secondary to inefficient *in vivo* transfection. Electroporation provides a means for transporting polynucleotides directly across the cell membrane into cells (Fig. 9). This process appears to enable the cytoplasmic entry of more of the delivered molecole compared to other nonviral methods. In addition, the electrophoretic effect may also facilitate intracellular transport to the nucleus once cytoplasmic uptake has occorre [31]. Extensive mechanistic research, mathematical modeling, and empiric development has been completed, and these studies provide significant insights into the basic mechanism of this procedure. Rational manipulations of electroporative parameters geared toward specific tissue features and laboratory conditions are possible. This gene delivery method relies on biophysical parameters rather than receptor expression, and therefore optimized methods appear to be easily transferred to enable transfection of similar cell types in different species. As a consequence, the successful use of electroporative nonviral gene transfer is rapidly becoming clinically feasible for a wide range of gene correction, gene therapy, and nucleic acid vaccine applications [32]. Furthermore, simple and efficient nonviral gene transfer methods like *in vivo* electroporation may also enable new opportunities for functional genomics research via direct somatic tissue transfection. To realize such oppor tunities, future research must address and overcome the mechanisms of toxicity, define the interaction between electroporative conditions and nucleic acid structure and size, and identify transcriptional elements that may be used to control the levels and distribution of transgene expression.

Irreversibile electroporation (IRE)

Irreversible electroporation (IRE) is in some applications the undesired, while in others it is the desired outcome of the electric pulse application. It is a consequence of membrane rupture that is a directly caused by electric pulse application. IRE and Joule heating are an integral part of electrical injury, which affects especially nerve and muscle cells due to their size. Release of intracellular components from affected cells cause acute renal failure due to deposition of iron-containing molecules such as mioglobin. Successful treatment of electroporated membranes with nontoxic polymers can reduce tissue injury produced by irreversible electroporation due to sealing of electroporated cell membranes. IRE is the desired result when it is used for microbial deactivation in water and food treatment. The applied electric pulses should cause irreversible damage of treated cells. For effective treatment, critical electric field parameters should be chosen properly. Typical pulse amplitude for microbial deactivation in water and liquid food is between 20 and 35 kV/cm, pulse duration, from micro- to milliseconds, and pulse number varies from ten to hundred pulses. For food preservation, amplitudes used are lower than for microbial inactivation in freshwater and liquid food. The main problem is the choice of optimal treatment parameters that would require minimal power consumption and would effectively disintegrate treated cells. Recently, irreversible electroporation was reported as an alternative minimally invasive surgical technique in medicine for tissue ablation of breast and pancreatic cancer [33-36]. For in vivo applications, mathematical models provided a valuable tool for proper electrode positioning and optimal pulse parameter determination for effective treatment [34].

Nanoelectroporation (nEP)

Typically, 1300 V/cm, 100 ls pulses were used for skin cancer trials. Most studies have used six or eight pulses with an interval of 1 s (1 Hz). For gene therapy, lower intensity and longer pulses, such as 125 V/cm, 25 ms; 200 V/cm, 10 ms have been found to be effective. Lately, ultra-short pulses of nanosecond

(ns) durations and 10–300 kV/cm (nsEP) have been used for electroporation. Under these conditions, the plasma membrane acts as a short circuit allowing the pulse to directly manipulate the internal organelles of the cell. Hallmarks of apoptosis, including phosphatidylserine translocation, and caspase activation have been observed with high-intensity nanopulse electroporation. With these effects, it is expected that the use of ultra-short pulses of sub-microsecond or nanosecond durations could be used for additional applications in biotechnology and medicine, specifically for cancer/gene therapy. The use of high-intensity DC pulses with durations shorter than the time constant of membrane charging (1 ls) can offer valuable insight into the biophysical mechanisms involved in the electropermeabilization of the cells [37]. However, due to the complexity of the design and the high cost involved in constructing a nanoelectroporation system, there is no commercially available nanopulsers.

ECT: EVOLUTION OF CLINICAL APPLICATIONS

First clinical trials

An overview of the clinical data on electrochemotherapy is provided in Tables 1 and 2 [38].

Table 1 – Clinical trials with electrochemotherapy on melanoma tumours

Reference	# of patients	# of nodules	Response				
			PD (%)	NC (%)	PR (%)	CR (%)	OR (%)
Bleomycin i.v.							
Rudolf[3]	2	24	1 (4)	1 (4)	0	22 (92)	22 (92)
Heller[18]	3	10	0	5 (50)	2 (20)	3 (30)	5 (50)
Mir[21]	7	30	1 (3)	2 (7)	3 (10)	24 (80)	27 (90)
Rols[5]	4	55	0	4 (7)	46 (84)	5 (9)	51 (93)
Sub-total	16	119	2 (2)	12 (10)	51 (43)	54 (45)	105 (88)
Bleomycin i.t.							
Glass[17]	5	23	0	1 (4)	4 (17)	18 (78)	22 (96)
Heller[22]	12	84	0	1 (1)	8 (10)	75 (89)	83 (99)
Gehl[8]	1	9	0	0	0	9 (100)	9 (100)
Rodriguez[9]	2	13	0	2 (15)	8 (62)	3 (23)	11 (85)
Byrne[12]	21	52	5 (10)	10 (19)	4 (8)	33 (63)	37 (71)
Kubota[7]	1	8	0	0	0	8 (100)	8 (100)
Sub-total	42	189	5 (3)	14 (7)	24 (13)	146 (77)	170 (90)
Cisplatin i.v.							
Sersa[27]	9	27	3 (11)	11 (41)	10 (37)	3 (11)	13 (48)
Cisplatin i.t.							
Sersa[23]	2	13	0	0	0	13 (100)	13 (100)
Sersa[28]	10	82	5 (6)	6 (7)	5 (6)	66 (80)	71 (87)
Sersa[16]	14	211	16 (8)	24 (11)	23 (11)	148 (70)	171 (81)
Snoj[30]	1	1	0	0	1 (100)	0	1 (100)
Sub-total	27	307	21 (7)	30 (10)	29 (9)	227 (74)	256 (83)
Total	94	642	31 (5)	67 (10)	114 (18)	430 (67)	544 (85)

The first report on the use of electrochemotherapy in treatment of cancer patients was by Mir et al. from Institute Gustave Roussy, Villejuif, France in 1991 and 1993. This phase I/II study was launched after preclinical studies demonstrating good antitumour effectiveness of bleomycin given intravenously and subsequent electroporation of the tumours, which is discussed in this issue in the article of L.M. Mir. In the clinical study 8 cancer patients with 40 head and neck squamous carcinoma nodules were treated. Objective responses were obtained in 72% and among these complete responses were obtained in 57% of the treated nodules. The tumour nodules that were treated with bleomycin only did not respond to the treatment. No side effects were reported, and even ripetitive treatment proved to be successful. This first clinical study demonstrated feasibility and also effectiveness of electrochemotherapy, as well as it indicated that electrochemotherapy may be effective also on tumours of different histological origin, due to the theoretical basis of electroporation principle for drug delivery. This was proved by other groups from Toulouse, France, Tampa, USA, and Ljubljana, Slovenia, which launched their own clinical trials on electrochemotherapy with bleomycin given intravenously on different tumour types, namely melanoma, basal cell carcinoma, and adenocarcinoma. New approach in electrochemotherapy, the use of bleomycin given intratumourally was introduced in 1996 by Heller's group from Tampa, USA.17 That study involved 5 patients with 23 melanoma metastases that were treated by electrochemotherapy with bleomycin given intratumourally. Very good antitumour effectiveness was observed. Objective response rate was 95%, with 78% of complete responses.

Altogether, these first studies using electrochemotherapy with bleomycin given either intravenously or intratumourally demonstrated that this treatment approach is effective on cutaneous tumour nodules of different histology with good local tumour control. Results of all clinical trials performed in five cancer centres that have been involved in electrochemotherapy, were summarised in 1998 by Mir et al. The results were gathered from Institute Gustave Roussy,

Villejuif, France, University of South Forida, Tampa, USA, Institute of Oncology, Ljubljana, Slovenia, Centre Claudius-Re´gaud, Toulouse, France and Institut Jean-Godinot, Reims, France. Clinical experience on electrochemotherapy with bleomycin given either intravenously or intratumourally on 291 cutaneous or subcutaneous tumour nodules in 50 cancer patients was reported. Electrochemotherapy was performed on tumour nodules originating from basal cell carcinoma, melanoma , adenocarcinoma, and head and neck squamous cell carcinoma. Objective responses were obtained in 233 (85%) of the 273 evaluable tumour nodules that were treated with electrochemotherapy, from these 154 (56%) tumour nodules were in complete response. This study clearly demonstrated that electrochemotherapy with bleomycin is effective in treatment of tumour nodules with different histology, and that the results of the treatment were comparable between the five cancer centres, in spite of small differences in treatment protocols that they used.

Table 2 – Clinical trials with electrochemotherapy on non-melanoma tumours

Reference	Histology	# of patients	# of nodules	Response				
				PD (%)	NC (%)	PR (%)	CR (%)	OR (%)
Bleomycin i.v.								
Belehradek[2]	HN SCC[a]	8	37	0	8 (22)	6 (16)	23 (62)	29 (78)
Mir[21]	HN SCC	13	77	21 (27)	8 (10)	15 (19)	33 (43)	48 (62)
Glass[4]	Basal cell carcinoma	2	6	0	0	4 (67)	2 (33)	6 (100)
Heller[18]	Breast adeno ca.	1	2	0	0	0	2 (100)	2 (100)
Domenge[19]	Breast adeno ca.	1	7	7 (100)	0	0	0	0
Domenge[19]	Salivary gland adeno ca.	1	20	0	0	0	20 (100)	20 (100)
Sersa[26]	Hypernephroma	1	1	0	1 (100)	0	0	0
Sub-total		27	150	28 (19)	17 (11)	25 (17)	80 (53)	105 (70)
Bleomycin i.t.								
Heller[22]	SCC	1	1	0	0	1 (100)	0	1 (100)
Panje[6]	HN SCC	8	8	0	2 (25)	2 (25)	4 (50)	6 (75)
Alegretti[32]	HN SCC	4	4	0	0	2 (50)	2 (50)	4 (75)
Rodriguez[9]	HN SCC	2	2	0	0	2 (100)	0	2 (100)
Burian[10]	HN SCC	12	12	0	0	2 (17)	10 (83)	12 (100)
Bloom[13]	HN SCC	54	69	0	30 (43)	22 (32)	17 (25)	39 (57)
Glass[20]	Basal cell ca.	20	54	0	0	1 (2)	53 (98)	54 (100)
Rodriguez[9]	Basal cell ca.	9	9	0	0	2 (22)	7 (78)	9 (100)
Heller[22]	Kaposi's sarcoma	1	4	0	0	0	4 (100)	4 (100)
Kubota[7]	Bladder transitional cell ca.	1	17	0	0	0	17 (100)	17 (100)
Panje[6]	HN adeno ca.	2	2	0	0	1 (50)	1 (50)	2 (100)
Rodriguez[9]	Breast ca.	2	14	0	0	6 (43)	8 (57)	14 (100)
Shimizu[11]	Chondrosarcoma	1	1	0	0	1 (100)	0	1 (100)
Sub-total		117	197	0 (0)	32 (16)	42 (21)	123 (63)	165 (84)
Cisplatin i.t.								
Sersa[23]	SCC	1	2	0	0	0	2 (100)	2 (100)
Sersa[23]	Basal cell ca.	1	4	0	0	0	4 (100)	4 (100)
Sersa[23]	Adeno ca. tubae	1	2	0	0	2 (100)	0	2 (100)
Rebersek[29]	Breast ca.	6	12	0	0	8 (67)	4 (33)	12 (100)
Sub-total		9	20	0 (0)	0 (0)	10 (50)	10 (50)	20 (100)
Total		153	367	28 (8)	49 (13)	77 (21)	213 (58)	290 (79)

a Head and neck Squamous Cell Carcinoma.

The next milestone was the introduction of cisplatin, a widely used chemotherapeutic drug, into electrochemotherapy protocol. The first reports on electrochemotherapy with cisplatin were by Sersa et al. from Institute of Oncology Ljubljana, in 1998. In separate publications the results on antitumour effectiveness of electrochemotherapy with cisplatin given intravenously or intratumourally on melanoma, basal cell, squamous cell and adeno carcinoma tumour nodules were presented. In the first study, application of electric pulses was performed on accessible melanoma tumour nodules in patients that were receiving standard intravenous cisplatin-based chemotherapy regimen.

The antitumour effectiveness of electrochemotherapy, i.e. application of electric pulses to tumour nodules together with cisplatin-based therapy was compared to the antitumour effectiveness of cisplatin-based therapy given intravenously alone. In 9 malignant melanoma patients, electrochemotherapy resulted in 48% objective responses of 27 treated nodules, whereas in 18 tumour nodules treated with cisplatin-based therapy 22% objective response rate of the tumour nodules was obtained. Furthermore, the median time to progression was longer in electrochemotherapy treated nodules (21 weeks) than in the cisplatin-based therapy treated nodules (4 weeks). It has to be emphasised that the treated tumour nodules in that study were much bigger than those treated in studies using bleomycin; therefore, it could be expected that the response rate was lower. However, no further attempt to introduce electrochemotherapy using cisplatin given intravenously was made, based on the good results obtained using electrochemotherapy with bleomycin, given intravenously and electrochemotherapy with cisplatin, given intratumourally.

The second study presented the results on electrochemotherapy with cisplatin given intratumourally in melanoma patients. The group from the Institute of Oncology Ljubljana reported results on 133 tumour nodules in 10 melanoma patients. Eighty-two tumour nodules were treated with electrochemotherapy using cisplatin given intratumourally, 27 tumour nodules were treated with cisplatin intratumourally, 2 tumour nodules were treated with electric pulses alone and 22 tumour nodules were untreated. The untreated nodules and the

nodules that were treated with electric pulses alone were subsequently treated with electrochemotherapy. A significant potentiation of antitumour effectiveness of cisplatin was demonstrated in electrochemotherapy treated nodules, whereas exposure of tumour nodules to electric pulses without cisplatin had no effect on tumour growth. Four weeks after therapy 78% objective responses were obtained in the electrochemotherapy group with 68% complete responses, and 38% objective responses were obtained in cisplatin group with 19% complete responses. At 124 weeks of follow up, a 77% control rate of tumour nodules treated by electrochemotherapy was observed, compared to 19% of those that were treated with cisplatin only. These data were confirmed by successive study on 14 melanoma patients where 211 tumour nodules were treated by electrochemotherapy with cisplatin given intratumourally. Objective response rate was 81%, with 70% of complete responses of the electrochemotherapy treated nodules. Based on these studies cisplatin was recognised as one of the two drugs that can be used in electrochemotherapy with intratumoural drug injection).

The presented studies form the basis for implementation of electrochemotherapy showing that electrochemotherapy can be used in tumours of different histological origin and that those two drugs, namely bleomycin and cisplatin can be successfully employed in combination with electroporation. It was also shown that bleomycin can be administered either intravenously or intratumourally while for the cisplatin, intratumoural administration results in better response compared to intravenous administration.

ESOPE study

This multi center study had thepurpose to evaluate and confirm efficacy and safety of electrochemotherapy with bleomycin or cisplatin on cutaneous and subcutaneous tumour nodules of patients with malignant melanoma and other malignancies [39]. This was a two year long prospective non-randomised study on 41 patients evaluable for response and 61 evaluable for toxicity. Four cancer

centers enrolled patients with progressive cutaneous and subcutaneous metastases of any histologically proven cancer. The skin lesions were treated by electrochemotherapy, using application of electric pulses to the tumours for increased bleomycin or cisplatin delivery into tumour cells. The treatment was performed using intravenous or intratumoural drug injection, followed by application of electric pulses generated by a CliniporatorTM using plate or needle electrodes. Tumour response to electrochemotherapy as well as possible sideeffects with respect to the treatment approach, tumour histology and location of the tumour nodules and electrode type were evaluated.

An objective response rate of 85% (73.7% complete response rate) was achieved on the electrochemotherapy treated tumour nodules, regardless of tumour histology, and drug used or route of its administration (Fig. 10). At 150 days after the treatment (median follow up was 133 days and range 60–380 days) local tumour control rate for electrochemotherapy was 88% with bleomycin given intravenously, 73% with bleomycin given intratumourally and 75% with cisplatin given intratumourally, demonstrating that all three approaches were similarly effective in local tumour treatment (Fig.11). Furthermore, electrochemotherapy was equally effective regardless of the tumour type and size of the nodules treated. Side-effects of electrochemotherapy were minor and acceptable, as reported by the patients. Conclusion:We demonstrated that electrochemotherapy is an easy, highly effective, safe and cost-effective approach for the treatment of cutaneous and subcutaneous tumour nodules of different malignancies. Electrochemotherapy can provide immediate clinical benefit in patients with advanced cutaneous and subcutaneous metastases.

European Standard Operating Procedures of ECT

The aim of this document was to define the standard operating procedures (SOP) in order to safely and conveniently treat, by electrochemotherapy, patients with cutaneous and subcutaneousnodules. This document provides the reader with the basis for understanding the mechanisms of the

electrochemotherapy aswell as its possibilities as antitumour treatment. It also has a decision chart to help the physician in choosing among the different treatment modalities reported in this SOP [40].

The Italian Experience

ECT has been introduced in Italy in 2005. So far only a few centers have been able to collect an adequate case load in order to evaluate the clinical impact of the procedure. Nowadays many centres are equipped with the Cliniporator pulse generator in Italy and are ready to start treating patients with ECT. We here report the clinical experience on the use of ECT gained so far in Italy, at the Torino and Padova University. Actually, up to now this new treatment approach has been applied mainly in patients with superficial metastases from different tumor types unsuitable for conventional treatments.

Authors from the Dermatologic Clinic of the University of Torino published a prospective nonrandomized study on 14 melanoma patients [41]. An objective response was obtained in 153/160 (95%) lesions, with a CR rate of 62%. No differences in response rate were observed between cutaneous and subcutaneous metastases. The lesion size was the most predictive parameter for response. Indeed, a response was obtained in 123/124 (99%) metastases sized ≤1 cm2 and in 30/36 (83%) larger lesions.

Another large, retrospective series was published by our group in 2009 [42]. The main aim of this study, which was the largest ever reported so far, was to evaluate not only the toxicity and the activity of bleomycin-based ECT performed according to ESOPE but also the response duration and the impact of ECT on disease-related symptoms and patients' functioning in everyday life by means of a dedicated questionnaire. It's crucial to assess whether this treatment is useful to improve quality of life, given that ECT application is mainly accepted, by the oncological community, in the palliative setting.

Antitumor activity was observed in all tumour types and both in melanoma and non-melanoma metastases (CR was obtained in 17/34 melanoma patients and in 9/18 non-melanoma patients). One month after the first ECT application, 125

out of 267 (47%) target lesions showed CR, 126 (47%) PR and 16 (6%) NC. As regards the factors influencing tumor response, it was observed an inverse correlation between complete local response and the maximum diameter of the target lesion: 66% for tumours <1,5 cm, 36% for nodules between 1,6 cm and 3 cm and 28 % for those > 3 cm. Tumor response does not appear to be affected by the drug administration route, as similar response rates were observed in patients who underwent ECT with intralesional, intravenous and combined bleomycin injection.

One month after the first ECT application an objective response was obtained in 50 of 52 patients (96%): in particular, according to RECIST criteria there were 26 complete (50%), 24 partial (46%) responses. In two patients (4%) there was no change in tumor size. Among 26 complete responders at the first ECT, 17 are locally disease free after a median follow-up of 9 months (range: 2-21 months).

The questionnaire for evaluation of patients' perception was completed in 36 cases (69%). The local disease-related complaints, if present, were the following: difficulty in sitting (as a consequence of treatment of large tumors on the back, n=4); walking (due to a tumor nodule on the foot, n=2); bathing (due to ulcerated disease, n=2); dressing (because of oozing, n=7); mastication (tumor nodule on the oral mucosa, n=1); pain (n=22). Thirty-four of 36 patients (94%) declared a positive impact of ECT on one or more of the items investigated before and after treatment (wound healing, bleeding, aesthetic impairment, activity of daily living, social relation, pain control). Nearly all patients (93%) experienced a local benefit, as demonstrated by the improvement of the scores assessing ulceration and bleeding, with a better aesthetics appearance stated by 31 of 36 patients. Among these patients, five with tumor nodules on the head and neck region indicated an improvement in aesthetic appearance at 1- and 2-months survey. Nine patients indicated an improvement in some of the activities of daily living as a consequence of tumour treatment and six in social relations. A better pain control was registered in 9 out of 22 patients with painful tumor nodules. Overall, 34 of 36

patients were satisfied of the treatment received, 1 uncertain, 1 unsatisfied; 34 of 36 (94%) patients were keen to further ECT in case of recurrence. The sum of the scores obtained in the six items (bleeding, ulceration, aesthetics, pain, activity of daily living, social relation) registered after ECT (at 1 month and 2 months) were statistically different as compared to that registered before treatment.

STUDY AIMS

The principal milestones of this study were the following:

- the evaluation of ECT antitumor activity, together with its toxicity and effectiveness in local tumor control, in different subgroup of cancer patients (melanoma, sarcoma, breast cancer, head and neck cancer)

- the introduction and implementation of a new pulse generator and new needle electrodes for ECT treatment to large and deep soft-tissue tumors

- the individuation of possible advancements to improve ECT delivery (in order to obtain a more precise electric field application to the tumor) or effectiveness biologists (in order to sensitize cancer cells to bleomycin or stimulate a systemic immune system reaction with ECT-induced tumor necrosis

- the study of TLRs levels of expression in tumor samples before and following ECT application to melanoma cancer patients

- the investigation of the sensitizing effect to BLM of L-Buthionine sulfoximine (BSO), a specific γ-glutamylcysteine synthetase inhibitor that blocks the synthesis of glutathione (GSH)

METHODS

PROJECT'S OVERVIEW

An overview of the milestones of this project id provided in Fig. 12. Four phase-II studies have been designed to evaluate ECT in the clinic: three of them have bee carried out and completed at the Veneto Region Oncology Research Institute of Padova to determine the activity and safety of ECT in melanoma (n=85), breast cancer (n=35) and sarcoma patients (n=34, in collaboration with the Rizzoli Orthpaedic Institute of Bologna). The fourth prospective study, focusing on ECT application to large and deep tumor, is still open and enrolling patients (currently, accrual has reached 20 of 35 patients). An additional retrospective study was performed, iun collaboration with the Institute of Oncology of Ljubliana, Slovenia, in 42 patients with head and neck cancer.

Gene expression analysis of TLRs in metastatic melanoma patients who underwent BLM-ECT treatment was performer in collaboration with the Surgery Branch of the Oncological and Surgical Sciences of the University of Padova.

In vitro cytotoxicity tests on melanoma cell lines (B16-F10 and SK-MEL-28) were performer through the WST-1 proliferation assay, in collaboration with the University of Ferrara, Department of Morphology and Histology.

PATIENTS' INCLUSION CRITERIA

In general, the patients enrolled in the clinical trials had superficial tumor nodules (not deeper than 3 cm), except for the study on deep-seated soft tissue tumors, in which the enrolled patients had tumor of 3-6 cm size and maximum 20 cm depth. Incluson criteria for each study are detailed in the respective paragraph within the Results section.

TUMOR REGISTRATION

Tumor nodules were registerd and theit largest diameter recorded, in accordance with RECIST (Response Evaluation Criteria in Solid Tumors) Criteria.

PATIENT MANAGEMENT

The anaeshesiologic management was choosen according to the anatomical site to be electroporated, disease extension and patient comorbidity and ranged from a local anaesthesia to a genral anaesthesia, passing through a general pharmacological sedation with Propofol, Fentany and Ketamine.

TREATMENT

Anesthesia

Local anestesia. A rectangular infiltration of local anaesthetic (Lidocaine, 2%, with epinephrine 0.5%) around the area to be treated, by injecting along 4 lines so that the nodule is ‘fenced in’ by local anaesthetic. If more than one nodule is to be treated, it is necessary to consider the total amount of lidocaine: for example, in a 70 kg patient the maximal dose without adrenaline is 210 mg (21 ml of 1% lidocaine or 10.5 ml of 2% lidocaine) and with adrenaline 420 mg (42 ml of 1% or 21 ml of 2%). This will set the maximum of nodules treatable by one session with local anaesthesia, not surpassing 3 mg/kg of lidocaine without adrenaline or 6mg/kg for lidocaine with adrenaline. If it is anticipated that there will be less than 0.5 cm from the tip of the electrodes to periost, local anaesthesia should be reconsidered, since it may be difficult to palliate the patient sufficiently. Another potential restriction to local anaesthesia is the localization of the nodules in previously irradiated areas: experience has shown that is seems more

difficult to apply local anaesthesia in these previously irradiated areas and if the option ‘‘local anaesthesia’’ is confirmed, it is recommended to put more anaesthetic and allow more time for lidocaine diffusion. Small cutaneous tumours may be anaesthetized by local infiltration of lidocaine just below the tumour, however, still covering the area of electroporation. This may increase the number of small nodules that can be treated with local anaesthetic in one session. Premedication with sedatives does not seem necessary.

Analgo-sedation. Analgesia is started at least 3 min before intratumoural injection: remifentanil 0.5 lg/kg bolus then 0.1–0.15 lg/kg/ min then adjusted to the response to the first pulses or target controlled infusion (target 2 to 4 ng/ml). It may also be replaced by alfentanil boluses 250–750 μg. Sedation is then started: propofol 0.5 mg/kg then 2–4 mg/ kg/h or target controlled infusion (target 1 to 2 lg/ml). Both are stopped at the last pulse. Post-operative pain is usually very low to moderate. Post-operative analgesia can be provided by intravenous paracetamol (1 g) and tramadol (100 mg), or paracetamol per rectum, as soon as the ECT started. It may be renewed every 6 hours until the 24th hour. Alternatively, instead of giving the paracetamol in a prophylactic way, it can be just offered to the patient after the procedure. No systematic prevention of nausea and vomiting is given but nausea or vomiting episodes can occur in a few sensitive patients. They spontaneously resume.

Drugs

Cisplatin

Intratumoural injection:

– dissolve to 2 mg/ml with sterile water

– protect from direct light

Injected dose is 0.25 ml (0.5 mg) per cm^3 of tumour tissue for tumours larger than 1 cm^3, and 0.5 ml (1 mg) per cm^3 of tumour tissue for tumours smaller than 1 cm^3 but larger than 0.5 cm^3. For tumours smaller than 0.5 cm^3 injected

dose should be 1ml (2 mg) per cm^3, as a somewhat larger loss to surrounding tissues would be anticipated (see Table 3 below).

Calculated tumour volume ($V = ab^2\pi/6$)	<0.5 cm^3	0.5–1 cm^3	>1 cm^3
Cisplatin dose (concentration: z2 mg/ml)	1 ml/cm^3 tumour tissue	0.5 ml/cm^3 tumour tissue	0.25 ml/cm^3 tumour tissue

Table 3. Intratumoral cisplatin dosages according to tumor volume

Bleomycin

Intratumoural injection:

Bleomycin distributed in vials each containing 15000 IU of bleomycin, determined by its activity. Previously, the term units has been used, where 15000 IU = 15 units. The recommended concentration of injection solution is 1000 IU/ml, dissolve in sterile water. • Injected dose should be 0.25 ml (250 IU) per cm^3 of tumour tissue for tumours larger than 1 cm3, and 0.5 ml (500 IU) per

cm^3 of tumour tissue for tumours smaller than 1 cm3 but larger than 0.5 cm^3. For tumours smaller than 0.5 cm^3 injected dose should be 1 ml (1000 IU) per cm3 (Table 4).

Calculated tumour volume ($V = ab^2\pi/6$)	<0.5 cm^3	0.5–1 cm^3	>1 cm^3
Bleomycin dose (concentration: 1000 IU/ml)	1 ml/cm^3 tumour tissue	0.5 ml/cm^3 tumour tissue	0.25 ml/cm^3 tumour tissue

Table 4. Intratumoral BLM dosages according to tumor volume

Intravenous injection:

The doses of bleomycin are low and there is not amandatory need for premedication. Bleomycin is infused at 15,000 IU/m^2. The bolus lasts not less

than 30 s but in not more than 1 min. After 8 min from BL infusion, allowing the drug to diffuse into tissues, electric voltages are administrated.

Electrodes

The appropriate electrode was choosen according to the tumor characteristics. If the tumour is less than 1 cm, consider using either plate or parallel array electrodes. In case the tumour is more than 1 cm, consider using the hexagonal array electrodes. Plates or needles depending on the location of the small nodule, respectively superficial or more deeply located (Fig. 13-14). Treating at a frequency of 5 kHz will reduce the number of contractions to one – however this contraction will be more forceful than if a frequency of 1 Hz is used. The use of the 5 kHz frequency is mandatory for the electrodes of type III (hexagonal geometry).

Post-treatment management

The patients are followe-up 4 weeks post-treatment or earlier if required. At that time, treatment efficacy can be determined in most cases. For larger lesions, more healing time may be necessary. Re-treatment can be considered upon the evaluation at 4 weeks post-treatment, but also later. A healing time of up to 10 weeks is admissible for lesions over 1.5 cm. For smaller lesions, healing time is in the order of 4–8 weeks.

TUMOR RESPONSE EVALUATION

Unidimensional, clinical or radiological, if reqired, in accordance with according to the RECIST criteria.

QUALIY OF LIFE ASSESSMENT

A pathology-dedicated questionnaire questionnaire was used. It was developed by means of physicians' and patients' interview, together with the creation of a

psychologist-guided patients focus group at our Institute, where most important disease' related symptoms and patients' reported outcomes were identified and discussed [42].

TUMOR TISSUE ANALYSIS FOR TOLL-LIKE RECEPTORS

TLRs study was performed on melanoma patients with in-transit metastases who underwent ECT. A first tumor tissue biopsy was collected before the ECT and another biopsy (of a not electroporated tumor nodule) was taken from the same patient after two days from ECT. Biopsies were immediately stored in liquid nitrogen until RNA extraction. We improved the extraction protocol in four consecutive steps because skin tissue was very difficult to process. The tested genes were the following: TLR3 (Toll-like receptor 3), TLR7 (Toll-like receptor 7), TLR9 (Toll-like receptor 9), TYR (Tyrosinase), GUS: beta-glucuronidase (housekeeping); B-actin: beta actin (housekeeping).

IN VITRO STUDY ON TUMOR SESITIZATION TO BLEOMYCIN

The aim of the study was to evaluate the effects of the pre-treatment with BSO in combination with the ECT on melanoma cell lines. The hypothesis was that the depletion of intracellular GSH levels induced by the BSO pre-treatment of cells, in association with the local delivery of chemotherapeutic drug by electroporation (EP) can enhance the antitumor effects of the drugs. Selected cell lines (*B16-F10 e SK-MEL-28*) were cultured in order to firstly define their proliferative rate. In a second step, the cells were treated with increasing dose of chemotherapeutic drugs (bleomycin or melphalan) to *identity the* IC_{50} (the drug concentration which inhibited cell viability by 50% compared to drug untreated control cells) from semi-logarithmic dose-response curves. Thirdly, the EP was introduced in experimental set up, in order to verify the enhancement of chemotherapeutic drug cytotoxicity, evaluated as reduction of the IC_{50}, compared to the absence of EP. After the identification of the dose-

response curves of bleomycin and melphalan on melanoma cell lines in the absence and in the presence of EP, the fourth step was to evaluate the effects of the pre-treatment of BSO in association with the subsequent drug treatment. Preliminary to this step is the analysis of the BSO effect on cell viability to exclude its cytotoxicity. Several doses of BSO were used to treat the cells, at different times. At the end of the treatments, cell viability was evaluated by using a spectrophotometric assay (WST-1). The identification of the optimal dose and pre-treatment time of BSO permits to perform the final step in which the ECT (EP + bleomycin or EP+ melphalan) was introduced after the pretreatment of the cells with BSO, in order to analyze and quantify the advantage obtained on anticancer activity as a function of BSO pretreatment, EP and their combination.

STATISTICAL ANALYSES

Clinical trials were planned following a two-stage design (specified in the Results section for each study) to optimize patient's accrual. Continuous data are presented as median (range). The association between parametric variables was assessed by means of the ANOVA on ranks test, Chi-square or Fisher's exact test, according to data. Local progression-free survival (LPFS) was the interval from response achievement until local disease progression or the last follow-up. Survival analysis was calculated using Kaplan-Meyer's method and the log-rank test was used for comparison. A P <.05 value was considered significant. Correlative analyses in the biological study were performed with Wilcoxon, T test and Chi-square test. Statistical analysis was performed with SigmaPlot Software, version 11.0 (Systat Software, Inc.).

RESULTS

CLINICAL ECT

MELANOMA [43]

Of 89 patients evaluated, one declined treatment, one had medical contraindications to ECT and two were deemed too ill to tolerate anaesthesia. Population and tumour characteristics of the remaining 85 patients are summarized in Table 4. The disease had relapsed in 15 patients (18 per cent) after isolated limb perfusion and 10 (12 per cent) after radiotherapy. Twenty-one patients (25 per cent) received conventional treatments at least 2 months after ECT: systemic chemotherapy in 13 and low-dose subcutaneous IFN in eight.

Table 4. Descriptive characteristics of 85 patients diagnosed with disseminated superficial melanoma and treated with 226 electrochemotherapy sessions

	No. of patients* (***n*** **= 85)**
Age (years)†	70 (35–93)
Sex ratio (M : F)	39 : 46
Body mass index (kg/m^2)†	27.6 (20.3–36.8)
TNM stage at enrolment in the study	32 (38)
IIIB	25 (29)
IIIC	28 (33)
IV	
Involved anatomical area	
Trunk	34 (40)‡
Upper limb	5 (6)
Lower limb	46 (54)
No. of skin metastases†	14 (1 to > 50)

Size of lesions (mm)†	11 (5–65)§
5	7 (2.6)
6–10	41 (15.3)
11–20	108 (43.3)
21–30	60 (22.4)
> 30	50 (18.7)
Confluent	2 (0.7)
Breslow thickness of primary melanoma (mm) †	3.10 (1.20–9.00)

*With percentages in parentheses unless indicated otherwise; †values are median (range). ‡Four patients also had metastases in head and neck region. §Total of 268 lesions. TNM, tumour node metastasis.

Electrochemotherapy. The median number of ECT courses per patient was 3 (range, 1–6). Twenty-four patients were treated with a single ECT, 15 with two, 30 with three, 15 with four, two with five and two with six procedures. Bleomycin was administered systemically, locally or in combination in 117, 17 and 92 sessions respectively. The median number of electrode applications was 45 (6–135) per session. Electric currents had the following parameters, according to the electrode type: a train of four to eight pulses of 400–730 V, 910–1000 V/cm, 100 μs duration and 5 kHz repetition frequency. The median registered electric current for a single application was 2.5 (0.6–9.2) A and pulse deliveries were adequate in 74 (87 per cent) of 85 patients.

Treatment toxicity .Post-treatment pain was manageable with minor analgesics in 78 (92 per cent) of 85 patients. No ECT-related serious adverse events were reported. One patient experienced sinus bradycardia under general sedation (G2) and the procedure was interrupted. Eleven patients experienced some postoperative complications including syncope in four patients, nausea/vomiting (G1–G2) in eight and fever in four (G1–G2). Hospital stay lasted a few hours for 60 of the 85 patients. A skin reaction (G3) was present in

15 (18 per cent), seven (8 per cent) and six (7 per cent) patients at the 1-, 2- and 6-month follow-up appointment respectively. None of the patient- or procedure-related factors analysed was predictive of severe skin toxicity.

Tumour response and patient outcome

Target lesions

One month after ECT, 129 (48.1 per cent) of 268 target lesions had achieved a complete response and 118 (44.0 per cent) a partial response; the disease was stable in 16 (6.0 per cent) and progressive in five (1.9 per cent).

Number of tumours

Overall, 894 lesions were treated: a median of 11 (1 to more than 50) lesions per patient, with a median size of 24 (3–75) mm. Three-hundred and ninety-three lesions (44.0 per cent) showed a complete response (Fig. 15), 492 (55.0 per cent) an incomplete response or stable disease, and nine lesions (1.0 per cent) had progressive disease. Among the 85 patients, 1 month after ECT there were 41 complete responders (48 per cent; complete response on target and non-target lesions and no new lesions) and 39 partial responders (46 per cent; complete or partial response on target lesions and partial response or stable disease in non-target lesions and no new lesions). In three patients (4 per cent), there was no change in tumour size and in two (2 per cent) tumour progression had occurred. As all metastases were electroporated, it was not possible to ascertain whether there was any ‘bystander effect’ of ECT on untreated skin nodules.

Local control and patient outcome

After a median of 3 (1–6) ECT courses, the 2-year local progression-free survival rate was 87 per cent. Follow-up lasted at least 2 years for 21 patients. Only six patients experienced recurrence within the ECT field, after a median interval of 16 (6–20) months. After a median follow-up of 26 (6–47) months, local tumour control (complete or partial response) was achieved in 42 of 48

surviving patients: a complete response in 33 patients and a partial response in nine. During follow-up, new lesions outside the treatment field appeared in some patients, but local control was maintained by either single or repeat ECT in 24 and 61 patients respectively. Of 41 complete responders to the first ECT, ten patients were locally disease-free after a median follow-up of 22 (6–42) months. Nineteen patients received a second ECT course to treat new lesions a median of 6 (2–14) months after the first treatment. Sixty-one patients underwent a second ECT course. Thirty of 61 patients experienced a local complete response and 31 had a partial response. Fifty-three (87 per cent) of these patients maintained local control after a median follow-up of 18 (7–37) months. Of the 39 patients who had further treatment after a partial response to the first ECT session, 19 showed a complete tumour response and 20 a partial response to the second course. In this subgroup of patients, a total of 168 lesions were retreated, of which 131 (78.0 per cent) showed a complete response and 37 (22.0 per cent) a partial response. Some patients affected by widespread skin metastases required up to three ECT cycles before obtaining a complete response and up to six cycles during follow-up to maintain local control.

At the end of follow-up, seven patients were alive and disease-free, 41 were alive with disease and 37 had died owing to disease progression. Survival was longer in patients with limb metastases than in patients with tumour spread on the trunk: median 30 (95 per cent confidence interval 7 to 49) *versus* 25 (3 to 33) months respectively ($P = 0.002$).

Combination of electrochemotherapy with other treatments

ECT was applied with limited toxicity in patients with disease relapse after either limb perfusion or radiation therapy. The combination of ECT and low-dose IFN also proved to be well tolerated (*Table S1of reference 43*).

Predictive factors for response

In multivariable analysis, a tumour size equal to or less than 3 cm and 20 or fewer lesions were associated with response to treatment. Prognostic variables for local control in the univariable analysis were the number of lesions, ECT cycles and electrode applications. Cox proportional hazards analysis showed that only increasing number of electrode applications at the first treatment (P = 0.041) and increasing number of ECT cycles (P = 0.005) were independent prognostic variables. Breslow thickness of the primary melanoma and location of the tumour lesion (limbs *versus* trunk; P = 0.002) were the best predictors of overall survival.

Discussion. Bleomycin is ineffective for systemic therapy of malignant melanoma. Local application of brief electric pulses enhances the delivery of bleomycin by temporary cell membrane permeabilization. In patients, ECT not only magnifies drug cytotoxicity but also produces antitumour vascular effects: transient vasoconstriction at the arteriolar level, which leads to drug retention in the tumour and also endothelial cell destruction. The synergism of these mechanisms explains the favourable results in the treatment of melanoma and non-melanoma skin cancers. The present findings confirmed that, even with repeated application, ECT is well tolerated, with limited, mainly dermatological, toxicity.

The effectiveness of ECT was demonstrated by the complete response at first application in almost half of 268 tumour lesions that were refractory to other treatments. This is comparable to most previous findings. The administration of a second ECT to patients with an initial partial response increased the local complete response rate to 78.0 per cent (131 of 168 nodules). The administration of up to six treatments resulted in a *2-year local tumour control rate of 87 per cent*. After a median follow-up of 26 months, only six (7 per cent) of 85 patients experienced a relapse within the ECT field. The median time to local recurrence in the ECT field was 16 months, thus ensuring disease stabilization in a palliative setting. Superficial local tumour control was achieved in 42 of 48 surviving patients. Although tumour control could have

been confounded by the administration of subsequent treatments, only a quarter of patients received additional systemic therapies; no differences in local control and survival were found between patients who received further treatments after ECT and those who did not (data not shown).

The outcome of patients with disease relapse after limb perfusion or radiotherapy and in patients receiving adjuvant low-dose IFN was analysed in regard to some previous results. An interesting, although preliminary finding, was the favourable outcome for these subgroups. This is important because of the concern that previous radiation could reduce bleomycin diffusion and electric field application. Finally, it could be intriguing to verify whether the antiangiogenic activity of low-dose IFN might improve the well known antivascular effect of ECT.

The association between tumour response and tumour size confirms previous observations of lower tumour response rates among larger tumours. The correlation between response and the number of tumour nodules raises the possibility that some lesions might have been electroporated beyond the recommended 20-min bleomycin washout. In the future, technical advances may allow the electroporation of more tumour lesions within the 20-min window.

The increasing number of electrode applications and increasing number of ECT cycles were the only predictors of superficial tumour control. This finding justifies multiple electrode insertions during a single procedure and the application of more ECT courses in patients with large and/or diffuse metastases. These technical issues might be overcome by the availability of newly designed electrodes.

In this series, patients with leg metastases were found to have a more favourable prognosis than those with lesions located in the trunk. No difference was observed between patients with superficial disease *versus* those with both superficial and visceral disease. This observation could influence the selection of patients for ECT; patients with few and small limb metastases, regardless of visceral disease, could be considered more suitable for treatment.

The present investigation has a number of limitations; it was carried out in a single centre, the patient cohort was relatively small, technical parameters were assessed only at the first procedure, and the study was conducted before the introduction of new therapies such as vemurafenib and ipilimumab. Nevertheless ECT proved to be highly active against metastases that were refractory to previous chemotherapy.

ECT leads to long-term stabilization of widespread superficial melanoma. From a clinical point of view, the present results suggest that patients with 20 or fewer skin metastases, smaller than 3 cm in diameter, particularly when confined to the lower limb, have a high local response rate, with disease control and favourable survival.

BREAST CANCER [44]

Patients' characteristics at baseline. Thirty-seven consecutive patients with refractory CWR were enrolled from December 2006 through September 2011. Two patients were excluded for the presence of a subcutaneous port-a-cath on the tumor-involved CW. Patients' characteristics, are listed in Table 5.

Table 5. Patient characteristics

Patient characteristics	
T stage of primary breast cancer	
T1 / T2 / T3 / T4	5 / 14 / 8 / 8
N stage	
N0 / N1 / N2 / N3	6 / 10 / 13 / 6
Lymphovascular invasion (%)	11 (31.4)
Time (months) to 1st recurrence[a], median (range)	31 (9-64)
Time (months) to 1st CW recurrence,	

median (range)	33 (9-58)
Time to referral[b] for ECT treatment	
median (range)	10 (3-45)
Age[c], median (range)	67 (35-88)
Karnofsy PS, median (range)	90 (70-100)
Patients with comorbidities[d] (%)	28 (80)
Clinical stage at accrual	
IIIB / IIIC / IV	6 / 5 / 24
Disease extension	
superficial / superficial plus deep	12 / 23
Size (mm) of skin metastases,	
median (range)	20 (10-220)
Type of metastases	
nodule / plaque	15 / 20
ER status positive[e]	24 (68.6)
PgR positive[e]	24 (68.6)
Her2 positive[f]	10 (28.6)
Triple negative	5 (14.3)
CW skin ulceration (%)	9 (25.7)
CW bleeding (%)	4 (11.4)

Abbreviations: **CW,** chest wall; **ER**, oestrogen receptor; **PgR**, progesterone receptor; **PS**, performance status

[a] Breast cancer recurrence in any site.

[b] Time interval from the diagnosis of skin metastases to the first visit to accrual in the study.

[c] Age at the time of ECT treatment.

[d] The most frequent comorbidities were: pain (12/35 patients); hypertension (11/35); upper limb oedema (10/35); fatigue (10/35); heart failure (9/35); chest wall skin ulceration. (9/35); anorexia (9/35); anorexia (7/35); diabetes (6/35).

[e] Oestrogen and progesterone receptors were considered positive with at least 30% of stained cell at the immunohisthochemical assay.

[f] HER-2/neu amplification was evaluated according to international guidelines [www.nccn.org, downloaded on
October 2006].

Electrochemotherapy. Overall, 62 ECT courses were delivered (median 2 ECT /patient, range 1-3). Fourteen patients (40%) received a single ECT, 15 (42.8%) received 2 ECTs and 6 (17.2%) 3 ECTs. The treatment was delivered according to the protocol in all cases and CW metastases were encompassed within the ECT-field. The procedure lasted a median of 25 minutes (range, 15-35) and the median hospital stay was 1 day (range, 1-3).

Treatment toxicity. No serious ECT-related adverse events were reported. One patient developed a G5 pulmonary embolism four weeks after the second ECT and died nine weeks thereafter as a consequence of respiratory distress syndrome; this event was judged as unrelated to ECT. After ECT, six patients (17.1%) experienced fever (G1-2) and 2 patients (5.7%) reported uncontrolled pain. Seven patients experienced other early complications, including nausea/vomiting (G1-2, n=4;), syncope (n=1) and urticaria (G1, n=1) that did not modify the clinical course.

Pain. After each ECT course, patients' reported pain scores improved in the interval between 1- and 2-month follow-up, although worsened with the increasing number of ECT courses. One week after the first ECT, 28 patients (80%) reported pain control ("no pain" or "mild pain") and after one month, this number rose to 33 (94%). One-week and one-month pain control after each ECT cycle is detailed in Figure 2.

Dermatological toxicity. A G3 skin ulceration was present in 5 (14%) and 2 (6%) patients at the one- and two-month control, respectively, following the first ECT (Fig. 3). These patients were among the 9 patients (25.7%) who already presented ulcerated skin metastases at the time of accrual. The percentages of patients with skin ulceration and/or pigmentation after each ECT

cycle are detailed in Figure 3. Finally, 3 patients (8.5%) reported a G1 transient alopecia, likely induced by systemic bleomycin administration.

Tumor Response (Target Lesions). A total of 516 metastases were electroporated (median 15, range 1-50), response was assessed on 196 target lesions (median size 20 mm, range 10-220). Two months after the first ECT, an objective response was reported in 32 of 35 patients (91.4%). Nineteen patients had CR (54.3%, 95% C.I. 38%-84%), hence satisfying the study primary endpoint. Thirteen patients (37.1%) had PR and three (8.6%) were stable. No local tumor progression occurred.

Among the 19 complete responders at first ECT, 10 patients were still local disease-free at a median follow-up of 32 months (range, 6–52). One patient, who locally relapsed after 16 months, was retreated, achieving a CR that was maintained at 12-month follow-up. Of the 9 remaining patients, only 6 received further ECT for NL (developed after a median of 8 months, range 3–14). After the second ECT, the local response was complete and partial in 4 and 2 patients, respectively. Five of these patients developed additional NL and have been scheduled for retreatment.

In the subgroup of patients (n=16) who reported a PR or SD, 14 developed NL. Seven patients underwent a single retreatment, while five patients (two with triple negative BC) required up to a total of three ECTs. At the end of the follow-up the local status was as follows: 5 CR, 6 PR, 1 SD, 4 progressive disease (PD). At the last follow-up, NL were present in 11 of 16 patients, so an "effective CW control" (CR or PR on electroporated metastases and absence of NL) was achieved only in 2 patients with triple negative disease.

There were no significant associations between patients' clinical-pathological characteristics and local response (Table 3). After a median follow-up of 32 months (range, 6-53), local tumor control (CR plus PR) in the electroporated metastases was obtained in 30 of 35 patients (85.7%, 22 with CR and 8 with PR).

Local progression-free survival. The median follow-up was 32 months (range, 6-53) and 3-year LPFS was 81%. In the five relapsing patients, the median time to local failure was 18 months (range, 9-22). LPFS was independent on the investigated clinical-pathological parameters.

New lesions-free survival. Overall, 23 of 35 patients (65.7%) developed NL (Fig.4b), after a median time of 6.6 months (range, 2.3-29.5). NL occurred in 9/19 (47.4%) of complete responders and in 14/16 (87.5%) patients who achieved PR/SD (*P*=.029). The NLFS was significantly related to the following parameters at log rank analysis: number of skin metastases, degree of tumor spread on the CW, response to the first ECT and type of systemic treatment post ECT. The number of metastases and the degree of tumor spread showed significant hazard ratios with the Cox proportional risk model.

An “effective CW control” (e.g. both local control and freedom from NL) was achieved in 12 of 35 patients (34.3%), by means of one (n=3 patients), two (n=8) or three ECT cycles (n=1) (Figure 6).

Overall Survival. Six patients (17.1%) developed distant metastases (median time 16 months, range 7-28.3). The estimated 3-year OS rate was 58% (median, 39 months). The OS was significantly higher in patients with longer disease-free interval from the primary BC (*P*=.047). There was no significant difference according to primary BC node status (*P*=.71) and disease extension (*P*=.65).

Discussion. The present study reports, for the first time, the prospectively collected data on 35 BC patients treated with ECT for CWR after mastectomy. In this experience, bleomycin-based ECT showed a significant activity in the treatment of metastases that were refractory to CW re-irradiation and different lines of systemic therapy, in fact more than half of the patients obtained a local CR at the first application. This finding is comparable with the results on BC

retrievable from mixed series of heterogeneous cancer patients treated with ECT. Remarkably, after a median follow-up of 32 months, only 5 out of 35 patients experienced a local failure (median time 17.6 months), resulting in a 3-year LPFS of 81%, that could be regarded as a satisfying CW control, at least in the palliative setting. We were not able to detect any difference in tumor control according to the patients' clinical-biological features, probably due to the small series, but also to the high ECT activity.

ECT antitumor effect is restricted to the volume of electrode application and this is documented by the occurrence of NL even in two thirds of our patients, a critical aspect that should be addressed in future clinical investigations. Notably, we reported a significantly lower incidence of NL in those with fewer and contained CW metastases together with patients who achieved CR. Also endocrine treatment after ECT was associated with a lower incidence of NL compared to systemic chemotherapy, but this finding could be explained by the intrinsic favorable disease natural history of endocrine receptor positive BC. Finally, HER2 expression did not impair the activity of ECT that remained an appreciable therapeutic option also in patients with more aggressive features (e.g. triple negative BC) to maximize the opportunity for local tumor control.

Although ECT showed satisfactory activity on refractory tumors, as many as 23 patients (65.7%) developed NL. We can desume that in these patients systemic treatments were not active, despite that ECT had a curative effect on the electroporated lesions. However, since after ECT the patients with more than 10 metastases received preferentially chemotherapy and those with 10 or less metastases endocrine therapy, further clinical investigation is needed to confirm our results, in order to select patients who are likely to achieve a CR after a single ECT and are less prone to develop NL, thus sparing them from repetitive treatments. According to our experience, retreatments with ECT should be limited to selected cases, due to the observation of increased patients' reported pain and risk of dermatological toxicity by the increasing number of applications. For instance, after the third ECT cycle no patient reported to be completely pain-free, either at one- or four-week assessment. Skin toxicity,

instead, could have also been favored by the variable pre-ECT radiation exposure (ranging from 72 to 116 Gy), in fact 9 out 35 presented with ulcerated metastases at the time of accrual. In this trial ECT did not improve patients' prognosis, but these negative data of survival in our opinion do not limit the applicability of this treatment in the daily practice because, also in presence of visceral metastases, ECT may reduce the morbidity of uncontrolled superficial disease spread. In this setting, the benefits of ECT are the shortness of treatment duration (the procedure lasts a maximum of 20-30 minutes), easiness of application, brief hospitalization (generally 1-2 days), high activity rates, repeatability, possibility of safe combination with other treatments and limited systemic toxicity. However, our work shed further light on some drawbacks that deserve consideration. First, ECT does not allow the treatment of lymph nodes or skin metastases near to an installed port-a-cath reservoir for intravenous chemotherapy, because the insertion of the needle electrode may injure nerves, vascular structures or the subcutaneous catheter. Second, more data on ECT early-, but also on long-term toxicity profile, are awaited. Third, the ECT treatment modalilities for CWR are not completely standardized, in fact the electrode insertion is operator-dependent and tailored to the clinically-detectable metastases. Fourth, there are no comparative studies with standard therapeutic approaches. Finally, since it is unclear if ECT provides a clinical benefit to all cases of CWR, there are no shared criteria for patients selection.

ECT is a promising treatment option for different tumors and has proved effective in the multidisciplinary treatment of advanced malignant melanoma, both by administering bleomycin or cisplatin. Surprisingly, the therapeutic properties of these drugs seems to be electroporation-improved also in BC, for which they have no or limited role in standard chemotherapy regimens. Recently, an increasing number of reports highlight the feasibility of ECT in this cancer. Although the clinical experience is smaller compared to melanoma, the preliminary results are encouraging. In a report from Cork Cancer Centre, it has been shown that ECT, by means of multiple applications, can locally

control widespread CWR. In the patient reported by Whelan et al, it is noteworthy that repetitive courses of ECT were well tolerated and metastases that were refractory to conventional therapies shrinked after ECT administration. In our first clinical study on 11 heterogeneous BC patients, we faced with different grades of superficial tumor spread on the CW. ECT proved to be active, but in the thin soft tissues of the CW after mastectomy, the needle electrode insertion was incomplete, consequently the applied electric voltages were often lower than that recommended for an effective electropermeabilization, raising some concerns on the feasibility of ECT in these patients.

More recently, the comprehensive ECT clinical experience on CWR from BC has been reviewed by Sersa et al . The authors analyzed eight clinical trials from 1996 to 2009, none of which was specifically designed for BC. From the collected data, the overall response rate was 89%, complete 59%. Although encouraging, these results were obtained by means of different treatment protocols, either for the chemotherapeutic agents (bleomycin or cisplatin) or their delivery route (intravenous or intratumoral). Finally, only 37 out 49 patients considered in this review were treated according to the European Standard Operating Procedures of Electrochemotherapy.

Radiation therapy remains the reference approach for the treatment of CWR. It allows a full thickness CW treatment as well as the simultaneous targeting of the lymph nodes. However, it is a common finding that a number patients are not suitable for a full dose treatment, due to previous radiation exposure or comorbidities, particularly when cardiotoxic agents are also used. In our opinion, these two treatment approaches may be not just compared, but also combined. Two intriguing preclinical studies from the Institute of Oncology of Ljubljana support a radiosensitizing effect of both electroporation-administered cisplatin and bleomycin in different types of tumors, so that the application of ECT followed by irradiation could be envisioned also in breast tumors.

In conclusion, although ECT does not enjoy enough evidential support to represent an alternative to conventional treatments for CWR from BC, in this series it proved active in re-irradiated tumors that were refractory to several systemic treatments. Unfortunately, BC deposits permeate CW dermal lymphatics and blood vessels and are prone to grow outside the electroporated areas, forcing therefore many patients to multiple ECT administrations. Although only approximately a third of patients (12 out of 35) achieved an "effective CW control", ECT has the potential to be applied earlier in the clinical course of CWR, in fact, according to our data, the patients with fewer, less scattered and complete responding metastases are less likely to develop NL, thus avoiding additional ECT administrations. The patients with tumor partial response or NL can still be managed with further ECT, but experience an increase of pain and dermatological toxicity which require a pressing refinement of the protocol for their clinical management.

SOFT TISSUE SARCOMAS (Abstract of the submitted paper)

BACKGROUND: Electrochemotherapy (ECT) had shown strong cytoreductive effect in soft tissue sarcomas (STS) animal models. The toxicity, activity and feasibility of ECT were explored in a two-stage, phase II clinical trial.

METHODS: Thirty-four patients with superficially-recurrent (maximum 3 cm-deep) STS of any histotype ineligible to conventional treatments received one to four cycles of intravenous bleomycin and percutaneous tumour electroporation, according to the European Standard Operating Procedures of Electrochemotherapy (ESOPE).

RESULTS: Tumour response after the first ECT, assessed on 71 target lesions (median size 4 cm, range 2-12 cm), was as follows: 32.3% complete, 52.9% partial, and 14.7% stable (Fig. 16). Fifteen patients received up to 4 ECTs for incomplete response or disease progression, although re-treatment did not significantly improve response rates (*P*=.205). Median follow-up was 19.3

months and two-year local control rate 72.5% (Fig. 17). Median time to progression was 5.1 months. Tumour response (*P*=.041) and control (*P*=.047) correlated with tumour grade. Toxicity consisted of manageable G3 skin ulceration and soft tissue necrosis in 35% and 23% of patients, respectively. The accuracy of electrode placement was 47.1%, the adequacy of electroporative current 85.3%.

CONCLUSION: ECT is a new treatment option for patients with recurrent STS. ECT technique deserves further investigation to contain local toxicity and improve tumour voltage delivery.

HEAD AND NECK CANCERS (Abstract of the submitted paper)

Objectives. To evaluate the effectiveness of electrochemotherapy (ECT) in the treatment of head and neck cancer.

Methods. Electronic records of patients treated with ECT between 2006 and 2012 were reviewed. They included patients with head and neck skin tumors (HNSTs) and oral cavity/oropharyngeal squamous cell carcinomas (OC/O-SCCs). Tumor characteristics and ECT parameters were tested for correlation with outcome.

Results. There were 42 patients. Three patients underwent cisplatin-ECT and 1 patient bleomycin (BLM)-ECT with plate electrode, the remaining 39 patients received BLM with needle electrode tumor electroporation and were included in the analysis. Twenty-seven (69.2%) had HNSTs, 12 (30.8%) OC/O-SCCs. Median tumor size was 35 mm. BLM was administered intratumourally, intravenously or in combination in 7, 7 and 25 patients after local anesthesia, sedation or general anesthesia in 16, 13 and 10 patients, respectively. Overall response was 59%, complete 38%. Complete response correlated with tumor presentation (primary 84%, recurrent 16%, *P*<.001), size (≤2 cm 73%, >2cm-≤ 4cm 26.3%, >4cm 14%, *P*=.022) and route of BLM administration (intratumoral 100%, intravenous 29%, *P*=.024). Basal cell carcinoma (BCC)

showed a trend for higher response compared with squamous cell and adenocarcinoma (75%, 26.3% and 33%, *P*=.071). HNSTs and OC/O-SCCs showed comparable complete response rate (41 and 33%, *P*=.734) and local control (1-year local progression-free survival [LPFS], 51 and 44% *P*=.887). Median LPFS was 12.5 months,

Local control was higher in patients with BCC (*P*=.029), and in patients who did not received previous chemotherapy (*P*=.027) or radiation (*P*=.063).

Conclusions. BLM-ECT is active in small-size, primary, HNSTs (particularly in BCC) and OC/O-SCCs. The patients with BCC and those who did not received previous chemotherapy or radiation achieve higher local control.

DEEP SOFT-TISSUE TUMORS (ongoing study)

The preliminary report of this ongoing study has includes 20 patients (of the 38 necessary by the study design) and is focused on feasibility and activity data.

Overall, the procedure seems more technically demanding, in fact the median time was 60 minutes (range, 25-90). This is due to the need of accurate electrode placement in deep tissues (under US guidance) during the procedure, and a slightly more management of the pulse generator to achieve a tailored electric field to each single tumor.

The response rate of ECT administered by means of the new pulse generator and long needle electrodes, was 100% in melanoma patients and 89% in non-melanoma patients (soft tissue sarcomas and other, rarer soft tissue tumors/metastases). Six melanoma patients maintained local control after 1-year follow-up.

IN VITRO STUDIES

TOLL-LIKE RECEPTORS INDUCTION

We collected the tumor speciment of 34 melanoma patients who underwent ECT for in-transit metastases. RNA levels of TLR-3, TLR-7 and TLR-9 between pre- and post-ECT were compared. There were no significant differences in gene expression pre versus post ECT treatment in metastatic melanoma patients (Fig. 18).

TUMOR SENSITIZATION TO BLEOMYCIN

Dose response curves were identified for both the cell lines (B16-F10 and SK-MEL 28) in presence of BLM alone and in presence of BLM plus electric pulses (EP) were calculated and also the IC_{50}. Of note, the association of EP to BLM decrease the IC_{50} in both cell lines (Fig. 19 and 20).

Pretreatment of melanoma cell lines with BSO, even at different concentrations, did not increase the citotoxicity of bleomycin and electric pulses (Fig. 21).

In parallel experiments, electric pulses have been tested also in combination with melphalan (L-PAM), an alkylating agent that is used in the treatment of melanoma. Interestingly, the association of the electric pulses seems to increase to cytotoxic action of melphalan on both melanoma cell lines (Fig. 22). Moreover, cell exposure to low concentrations of L-PAM, in presence of both EP and BSO pre-treatment, was associated with the higher cytotoxicity in SK-MEL 28 cell line (Fig. 23).

DISCUSSION

ECT proved to be an active antitumor treatment in all the tumor histotypes in which was tested (melanoma, breast cancer, soft tissue sarcomas, head and neck cancer). The overall response rate was 94, 91, 85, 59%, respectively.

This promising activity data translated in variable outcomes as to local tumor control, according also to disease stage and the underlying prognosis. Many patients with skin metastase, infact, have a dismal prognosis due to high tumor aggressiveness and when tumor skin infiltration occurs, have generally hexausted multiple treatment options.

Hence, patients selection is of paramount importance in a treatment, such as ECT, that is mainly applied in advanced stage disease patients. In this way, our findings from melanoma and breast cancer patients, could help, to individuate possible reliable predictive factors in order to select for ECT only the patient who benefit most from it. The collection of patients' functional outcome together with patients' quality of life data will give a useful and complementary point of view, especially when the rate between benefit and morbidity is not yet clearly established, as in head and neck cancers treatment.

However, even thanks to the possibility of being easily repeated, ECT ensured the sustained local progression-free survival rates, ranging from 81 to 87%. Survival data in head and neck cancer are more controversial and, up to now, homogeneous study populations are lacking. However, our preliminary experience showed the feasibility of treatment application also to mucosal squamous cell carcinomas of small size and located in the proximal side of the oral cavity and oropharynx..

Through our phase II trial performed on large and deep soft tissue tumors, we showed that the combination of electric pulses and chemotherapy works also on

big size tumors, even thouth different pulse parameters with higher amplitude are necessary and a specific training for the treating physician is mandatory.

Possible strategies for improving ECT effectiveness have been explored, i.e. immune system reaction (in 34 melanoma patients) and tumor sensitizing to BLM by means of BSO pre-treatment (in melanoma cell lines).

Contrary to a preclinical report on an animal model, we were not able do demonstrate an induction of the TLRs RNA in tumor biopsie performed 48 hours after ECT treatment, however a diffuse lymphocyte infiltration was associated with a higher response rate. Although the patients respected a wash-out from other treatments before ECT, the heterogeneity and extent of previous anticancer therapies may have influenced our results.

Finally, BSO pretreatment seems not to sensitize two melanoma cell lines to BLM. On the contrary and interestingly, it appears to increase the cytotoxic action of melphalan, particularly when in association with electric pulse. This findings open the possibility to explore melphalan chemotherapy, already used in the setting of the isolated limb perfusion, in combination with tumor electroporation and BSO pre-treatment. Further in vitro experiments are necessary to individuate possible ideal treatment parameters in order to move to clinical tests in melanoma patients.

ACKNOWLEDGMENTS

I wish to thank my Colleagues from Radiotherapy Section (*Sara Galuppo, Michela Basso, Luigi Corti, Guido Sotti*), with whom I shared most of the ECT procedures.

An earnest thanks to all the anesthesiologists (*Sandra Cappellato, Connie Celentano, Angelo Ciccarese, Elisa Granziera, Muzio Meroni* who have steadily improved the anesthesia care, ECT by ECT; without them we would not have been able to treat and manage adequately our patients.

I have to thank *Loris Bertazza* for his help with the processing of the tissue samples and for having the patience to set up an effective protocol for the processing of skin tumor biopsies.

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I apprciated the collaboration with *Prof. Fabrizio Dughiero* and *Elisabetta Sieni*, from the Faculty of Engineering, who have the patience to teach me some notion of physics.

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brought fruitful inspirations and contributed,

in various extent, at this project.

Thank you all!

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FIGURES

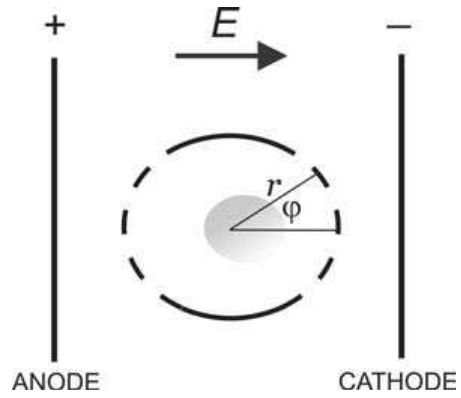

Fig. 1 Cell in an electric field. The induced transmembrane potential is maximal at the poles of the cell in accordance with Schwan's equation (Introduction section). Electroporated area is presented with dashed line.

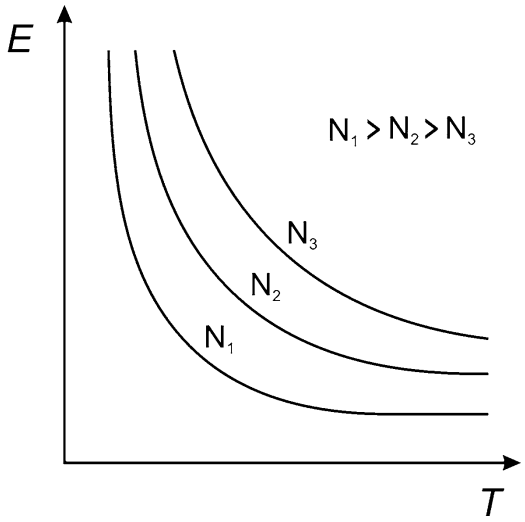

Fig. 2 Fraction of electroporated cells is increasing with increasing number of applied pulses. Abbreviations: E, electric field strength; T, pulse duration, N, number of applied pulses

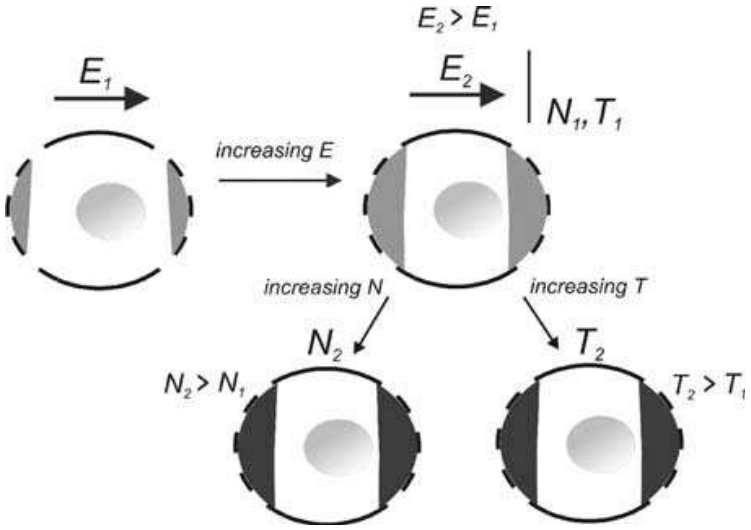

Fig. 3 Increasing the pulse amplitude results in larger area of membrane with smaller extent of electroporation, while increase in pulse number or duration does not affect the membrane area but increases the extent of electroporation

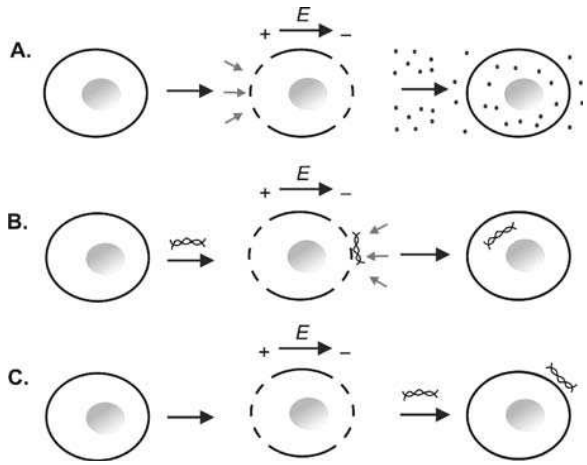

Fig. 4 Introduction of small and large molecules by electroporation. (**A**) Introduction of small molecules takes place during and predominantly after the pulse. Electroporation of the cell membrane is asymmetrical and occurs first at the anode side (small *grey* arrows). (**B**) Introduction of DNA into the cell. DNA must be present before electric pulses are applied. The initial step is DNA adsorbtion to the cell membrane, which takes place in the cell membrane facing cathode (small *grey* arrows). (**C**) When DNA is added after the pulse application it cannot be introduced into the cell.

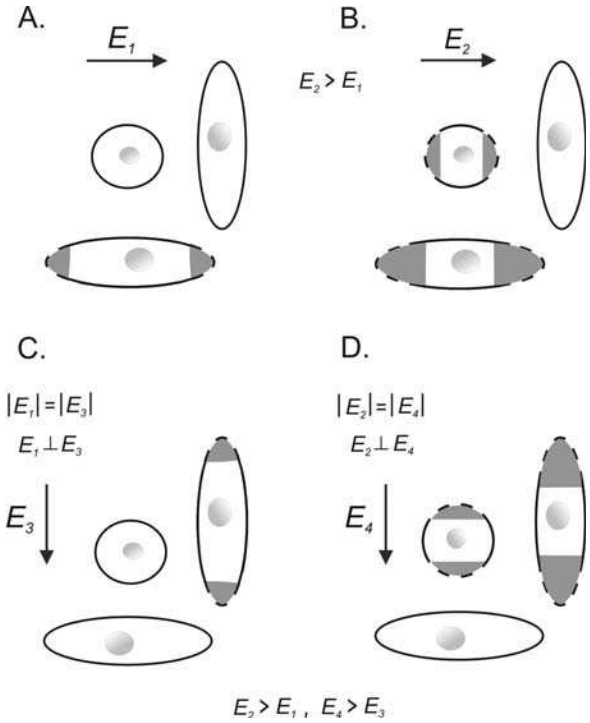

Fig. 5 Effect of electric field orientation on electroporation of different cell sizes and shapes. (**A**) Electric field parallel to elongated cell. (**B**) Electric pulse amplitude is increased. (**C**) Orientation of electric field is changed. (**D**) Electric pulse amplitude is increate.

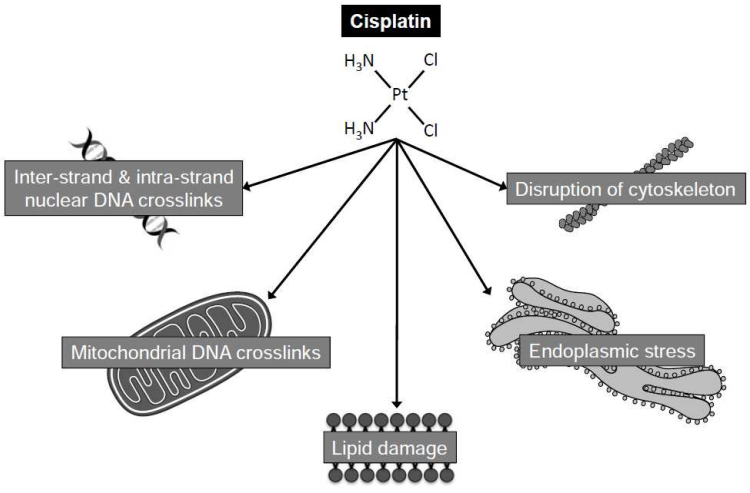

Fig. 6 Formation and effects of cisplatin. Cisplatin undergoes aquation to form [Pt(NH3)2Cl(OH2)]+ and [Pt(NH3)2Cl(OH2)2]2+ once inside the tumor cell. Cisplatin induces inter- and intra-strand nuclear DNA crosslinks, binds to mitochondrial DNA, causes endoplasmic stress, interacts with phospholipids

and phosphatidylserine in membranes, disrupts the cytoskeleton and affects the polymerization of actin. These biological consequences are responsible for tumor cytotoxicity.

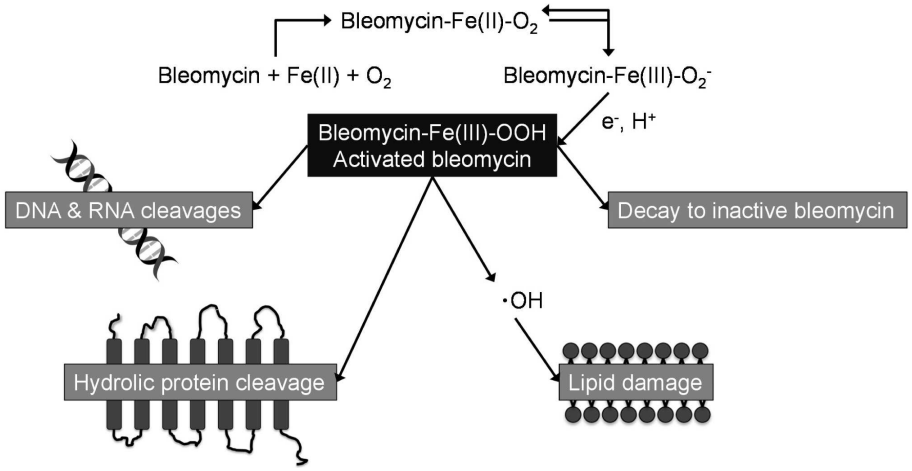

Fig. 7 Formation and effects of activated bleomycin. Activated bleomycin can be formed by bleomycin binding to Fe(II), followed by oxygen binding and reduction by a reductant. This intermediate (black box) has a half-life of several minutes at 4°C. It can destroy itself, oxidize phospholipids, hydrolyze amide bonds of proteins and initiate chemical and sequence-specific cleavage events on RNA and DNA. These biological consequence and dsDNA cleavage are responsible for tumor necrosis.

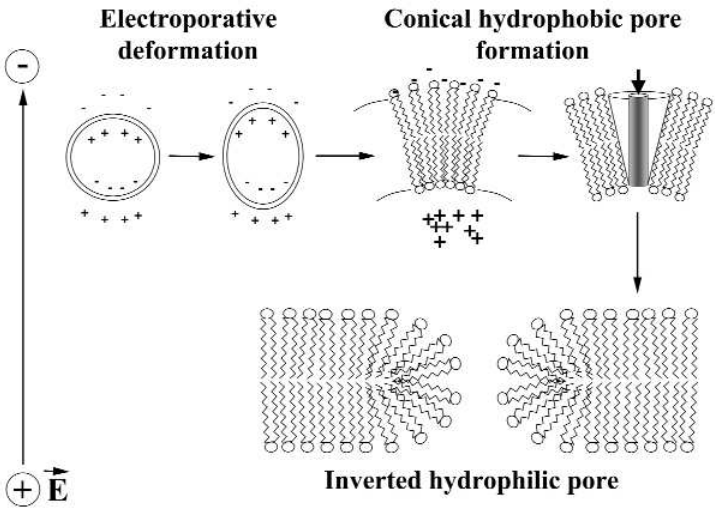

Fig. 8 Model for electroporative channel development. Under the influence of an electrical field, the distribution of ions adjacent to the inner surface of cell membranes is proposed to be altered, resulting in a series of membrane alterations that predispose to pore formation.

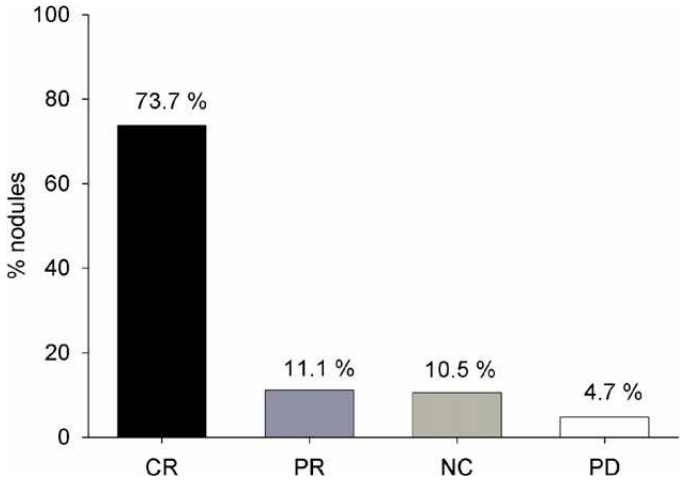

Fig. 9 Therapeutic efficacy of electrochemotherapy on cutaneous and subcutaneous tumour nodules (n=171) of different histology in 41 patients (results of the ESOPE study).

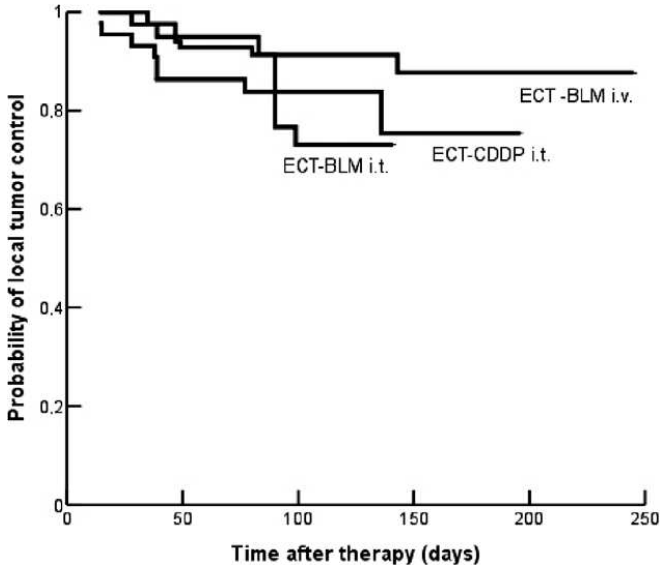

Fig. 10 Local tumour control curves for ECT with bleomycin given intravenously (ECT–BLM i.v.; 86 nodules) or intratumourally (ECT–BLM i.t.; 41 nodules), and cisplatin given intratumourally (ECT–CDDP i.t.; 44 nodules). Local tumour control rate was estimated with the Kaplan–Meier method, and the difference between the curves was analyzed by means of the log-rank test.

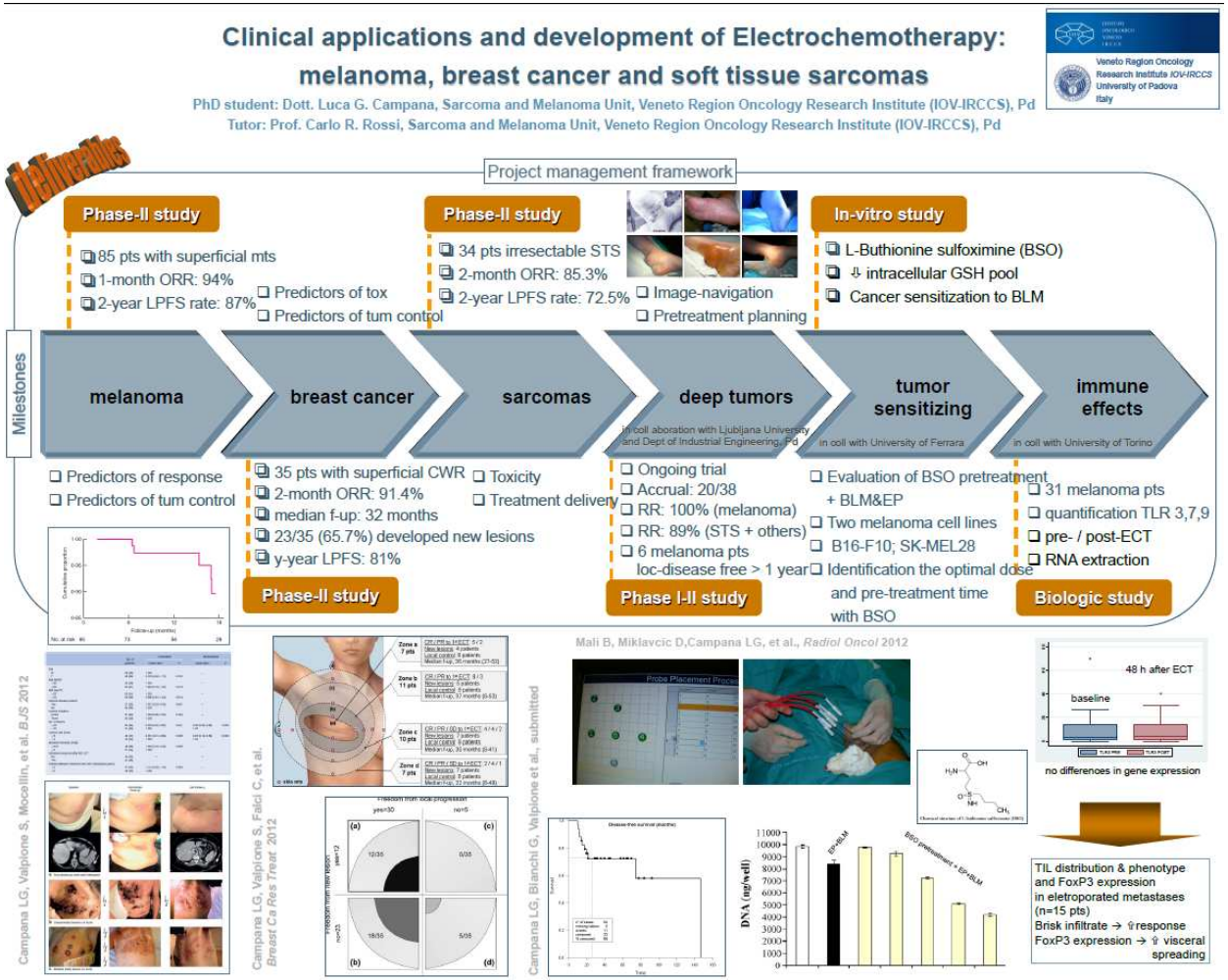

Fig. 11 Electrochemotherapy project overview with milestones and deliverables

Fig. 12 Eletrode models: plate (left), needle with linear array (in the middle) and needle wiyh an hexagonal array.

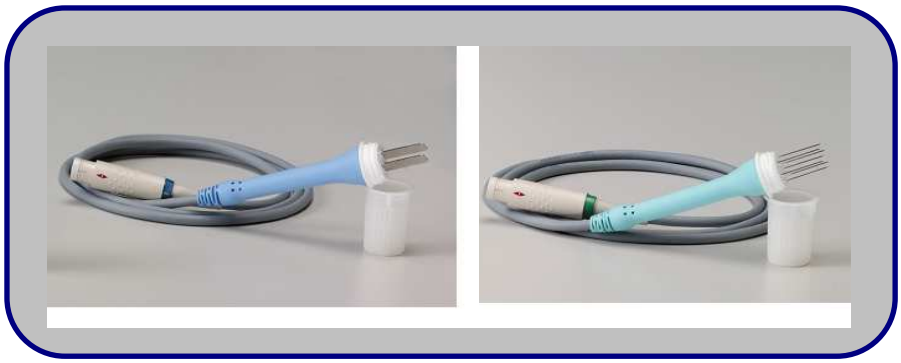

Fig. 13 Electrode connected with their handle

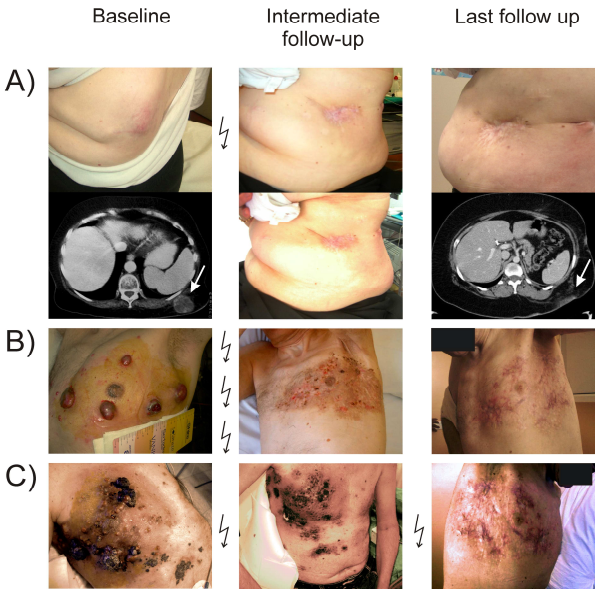

Fig. 14 Cutaneous metastases in three patients treated with electrochemotherapy (ECT). **a** Large subcutaneous recurrent metastasis of the chest wall in a patient previously treated with wide surgical excision (left panel; baseline computed tomogram shown below). A complete response was observed 2 months after a single ECT session. Intermediate clinical follow-up was at 6 months (centre panel). Additional cytotoxic chemotherapy and interferon therapy was administered for lung metastases after 18 months. The patient was disease-free at the last clinical and radiological examination at 42 months (right panel). **b** Disseminated lesions of the trunk at baseline (left panel). Treatment with ECT led to a partial response at intermediate follow-up at 1 month (centre panel). There was complete and long-lasting cutaneous remission after the second ECT cycle, and the patient was alive with disease at last follow-up after 37 months (right panel). **c** Multiple bulky lesions of the trunk at baseline (left panel). Treatment by three ECT cycles led to a complete response at intermediate follow-up after 12 months (centre panel) with superficial tumour control at 42 months (right panel). Each jagged arrow (ϟ) represents a single ECT session.

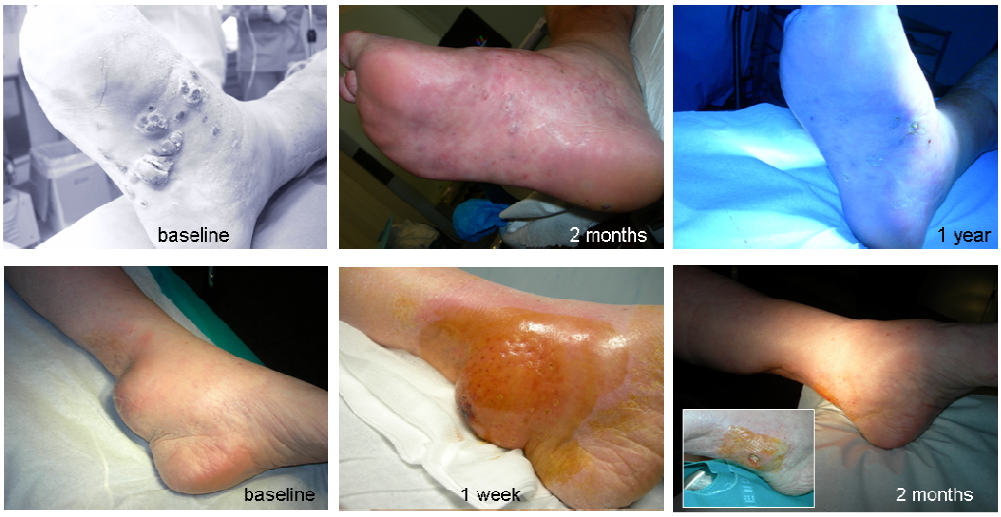

Fig. 15 Examples of complete response after electrochemotherapy in soft tissue sarcoma patients (Kaposis's sarcoma and leiomyosarcoma).

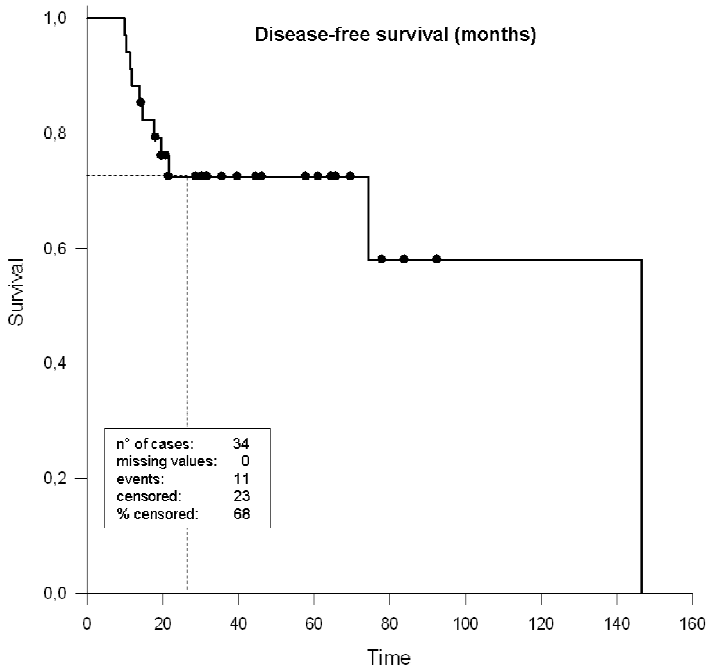

Fig. 16 Kaplan-Meier estimate of local progression-free survival after ECT in patients with recurrent soft tissue sarcomas.

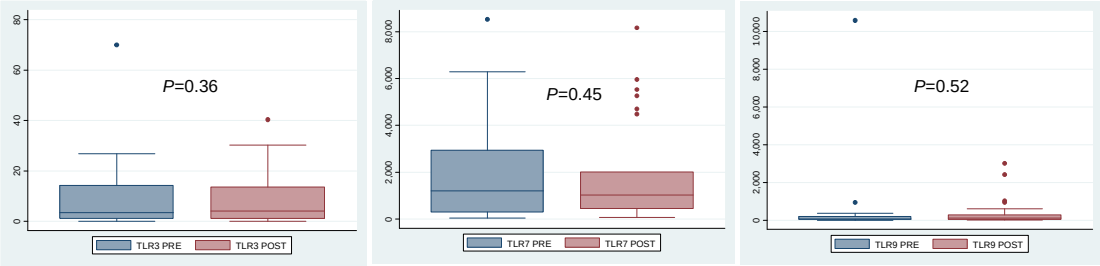

Fig. 17 TLR-3, TLR-7, TLR-9 RNA expression levels in cutaneous metastases of melanoma patients, sampled before and after ECT* (at 48 hours) in 34 melanoma patients. * The tumor biopsied at 48 hours was not electroporated during ECT session.

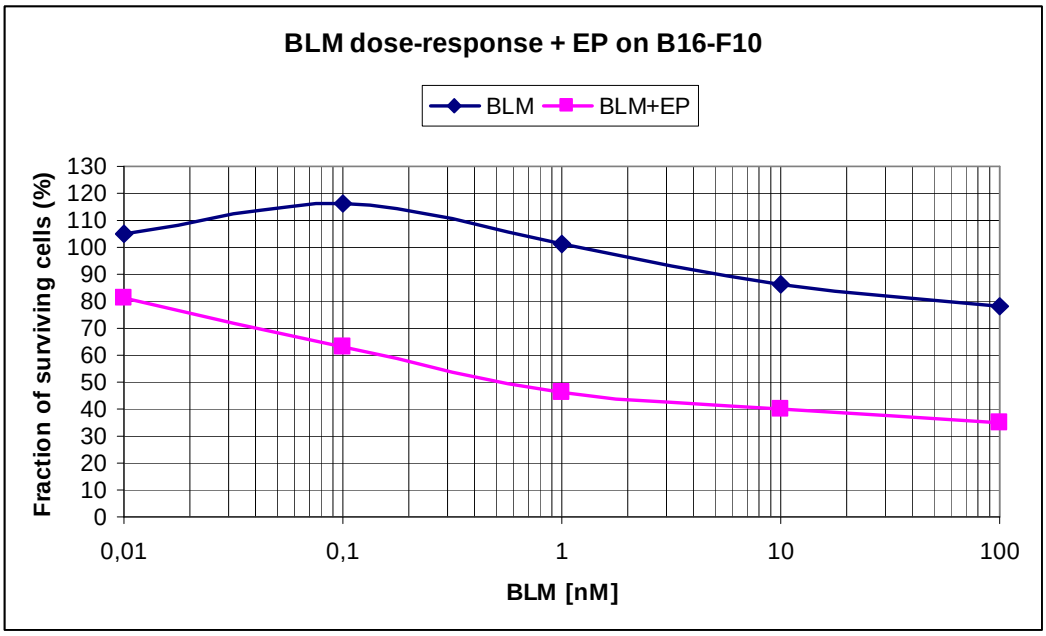

Fig. 18 Dose response-curve of B16-F10 melanoma cell lines in presence of bleomycin alone or after the combined treatment of bleomycin and electric pulses. IC_{50} BLM >> 100 nM; IC_{50} BLM+EP= 0.5 nM

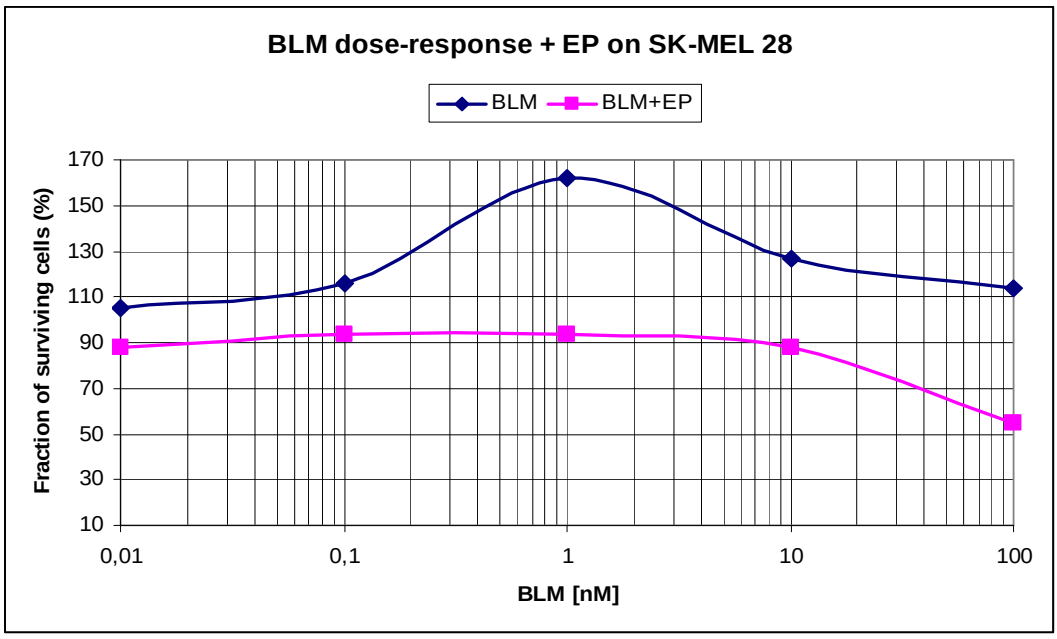

Fig. 19 Dose response-curve of SK-MEL 28 melanoma cell lines in presence of bleomycin alone or after the combined treatment of bleomycin and electric pulses. IC_{50} BLM >> 100 nM; IC_{50} BLM+EP= 100 nM

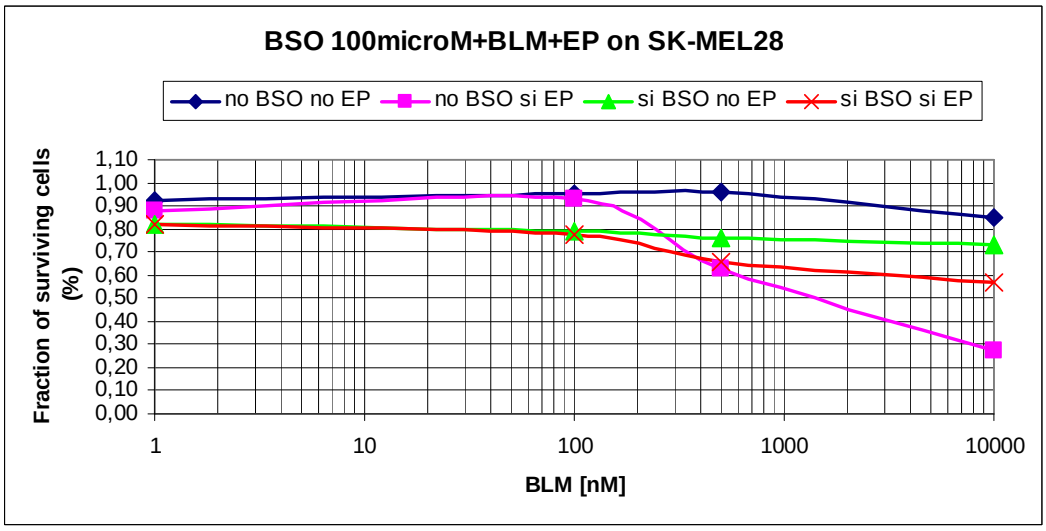

Fig. 20 Dose response-curve of SK-MEL 28 melanoma cell lines exposed to bleomycin in four different settings:

- no BSO pretreatment and no electric pulses (EP)
- no BSO pretreatment, si EP
- si BSO, no EP
- si BSO, si EP

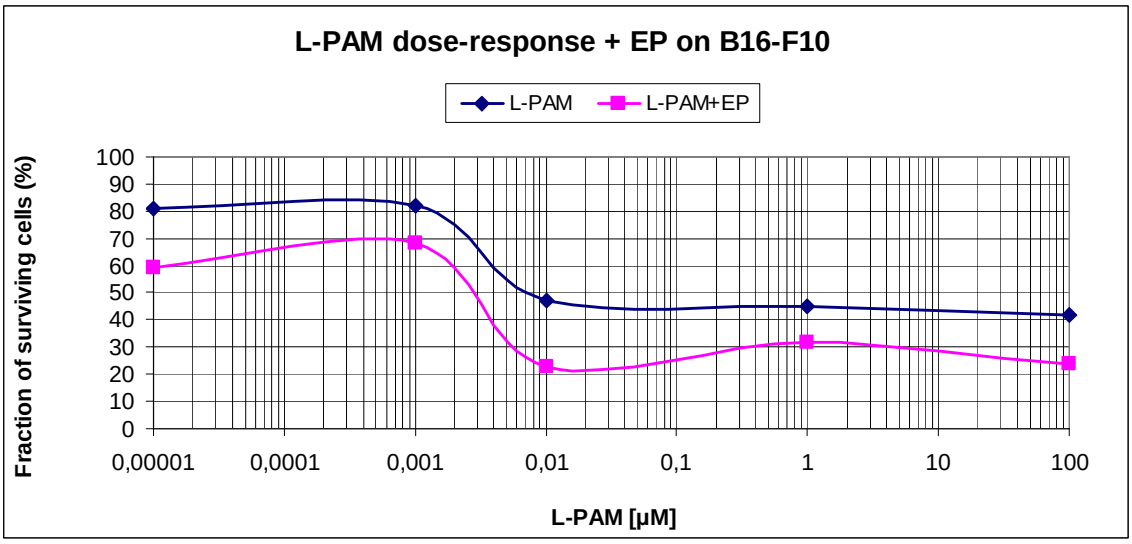

Fig. 21 Dose response-curve of B16-F10 melanoma cell lines exposed to melphalan (L-PAM) alone and L-PAM plus electric pulses.

IC_{50} L-PAM=2.7nM; IC_{50} L-PAM+EP=7nM

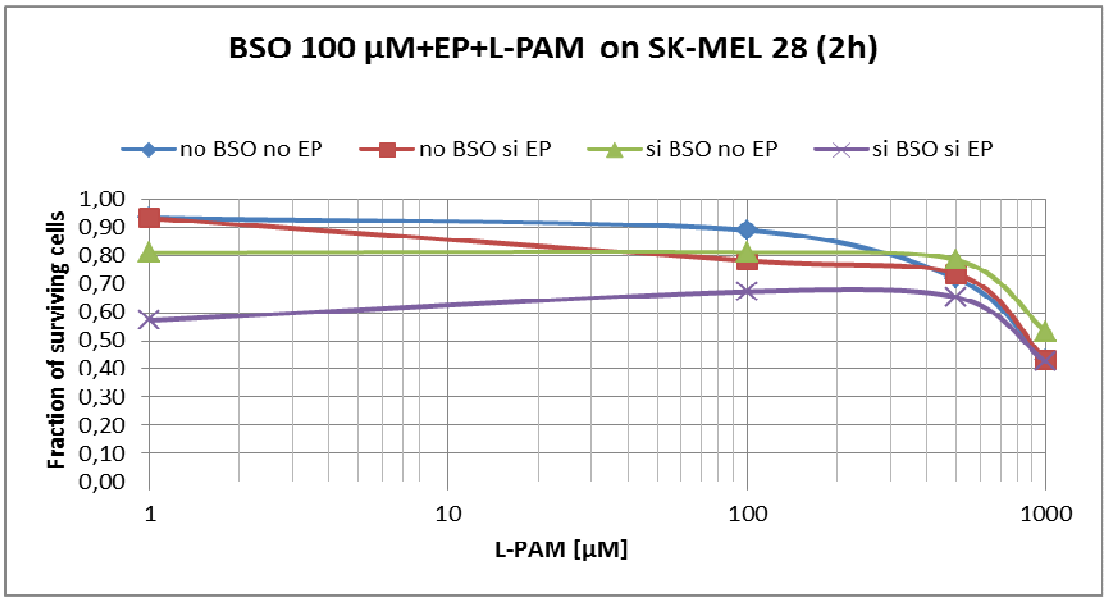

Fig. 22 Dose response-curve of SK-MEL 28 melanoma cell lines exposed to L-PAM (for 2 hours) in four different settings:

- no BSO pretreatment and no electric pulses (EP)
- no BSO pretreatment, si EP
- si BSO, no EP
- si BSO, si EP