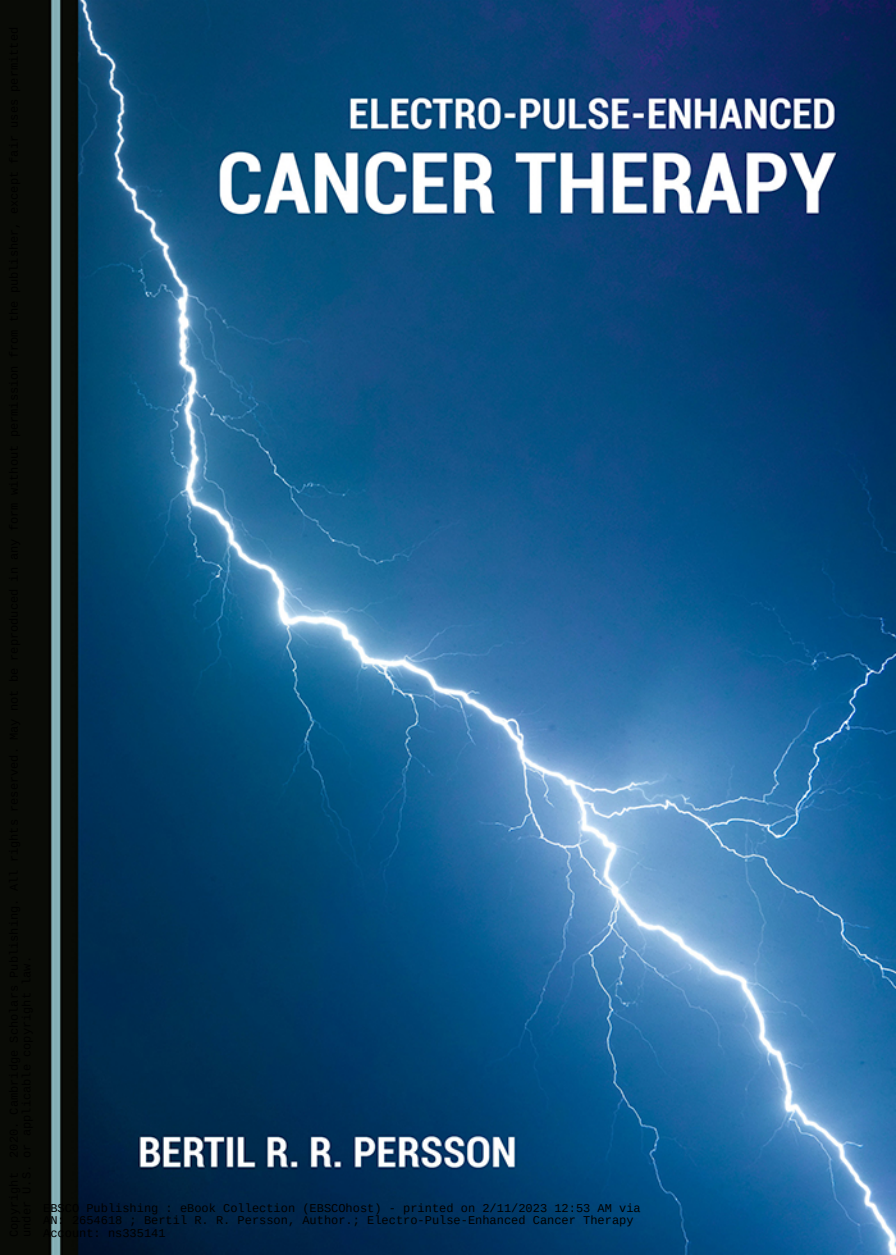

Electro-Pulse-Enhanced Cancer Therapy

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By

Bertil R.R. Persson

Cambridge Scholars Publishing

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ABSTRACT

This book is a summary of my 30 years' experience of electro-pulse-enhanced-therapy in the treatment of malignant tumours.

The first chapter is a short history of the discoveries that led to the clinical use of electro-pulse-enhanced cancer therapy (EpECT). In the late 1960s, Sale found that the action of inducing short electric pulses upon biological cell membranes made them transiently permeable without damaging the membrane structures. In the 1970s, Zimmermann observed that red blood cells began to lose haemoglobin after exposure to a pulsed electric field that exceeded a certain threshold. Exposure to short electrical pulses with amplitudes between 4 and 6 kV/cm resulted in the cells melting together into larger cells containing several nuclei. In the 1980s, Neumann and Wong demonstrated that electrical field-mediated transfer of DNA into cells is a handy technique, simple and easy to apply for DNA transfection. Okino and Mohri reported in 1987 the possibility of using the electroporation effect for enhanced transport of the anticancer drug Bleomycin across the cell membrane. The first clinical trial with EECT was performed on head and neck tumours by L. Mir and colleagues in France, 1991.

The second chapter describes the electrochemical and biophysical principles of the action of high voltage electrical pulses applied to human cells and tissues. The creation of channels in the cell-membrane, usually called electroporation, is the main phenomenon considered in electro-enhanced chemotherapy. However, electrochemical processes may also be involved.

My profession is in medical radiation physics, where radiation dosimetry is the most critical issue in radiation therapy. Thus, I considered it essential to know for certain the amount of energy delivered to the patient in electro-pulse enhanced cancer therapy as well.

The third chapter describes the measurement of the change in the electrical impedance of the tissue as an excellent way to estimate the energy delivered to the tissue through exposure to electrical high voltage pulses.

Another branch of my profession is the use of radio-pharmaceuticals to study the morphology and function of various human organs. As such, the fourth chapter describes the use of radioactive labelled drugs and gamma-camera recording to explore the enhanced uptake of drugs after exposure to electric pulses. Multivariate statistical methods are used to predict the

dependence of drug uptake in the exposed and non-exposed tissue on several variables

The fifth chapter summarizes the results of numerous preclinical studies of electro-pulse enhanced cancer therapy. Bleomycin and Cis-platinum are the drugs mainly used in clinical applications of such therapy. The results of extensive preclinical studies are shown to indicate promising combinations of electro-pulse chemotherapy with established immune therapy and radiation therapy regimes.

As mentioned above, the first clinical trial using Bleomycin and electric pulse treatment (called “electro-chemotherapy” (ECT)) was performed on head and neck tumours by Mir and colleagues in France in 1991. In the following years (up until 2018), clinical trials have treated more than 1500 patients with malignant melanoma, Kaposi´s sarcoma, breast cancer, squamous cell carcinoma, and basal cell carcinoma. The sixth chapter reviews and analyses the clinical results of these trials.

The seventh section discusses the safety concerns that must be taken into account when treating patients with high voltage electrical pulses.

The eighth, and final, part of the book presents a summary of potential new dimensions in electro-pulse enhanced Cancer therapy.

ACKNOWLEDGEMENTS

This book is dedicated to my collaborators in the field of electro-pulse-enhanced cancer therapy (EpECT) over the past 30 years: Leif G. Salford, MD, Professor of Neuro Surgery at Lund University, who opened his laboratory for the first experiments with malignant glioma implanted in rat brains; Louis Mir who supported us with a French electroporation device for animal experiments which led to the publication of "A new brain tumour therapy with EpECT"; Per Henriksson for the "Zero Weed" adventure; Maria Danfelter MSc, for her dedicated pioneering work with cell survival; Per Engström, PhD, for his eminent pre-clinical pioneering work, a strong thesis, and for conducting the first clinical operations with EpECT; Catrin Bauréus-Koch, PhD, for her dedicated work in the extensive rat experiments using radiation and immune therapy combinations, and her exquisite thesis; Gustaf Grafström, PhD, for his skilful operations in the extensive rat experiments; Susanne Strömblad and Catarina Blennow, for their skilful technical assistance in the animal experiments and histology preparations; Bernt Böhmer, engineer and entrepreneur, for his skilful constructions and manufacturing of our various electro-pulse devices; Anders Johnsson, for promoting the gamma camera experiments, and Mohan Frick for bringing electro-pulse-enhanced cancer therapy to the market.

The authors wish to thank the QuickField support team for the temporary license of the program, Version 6.3.2 (Tera Analysis Ltd. http://quickfield.com), without which this work summarized in chapter 3.5 could not have been performed.

CHAPTER I

INTRODUCTION

1.1 ELECTROPORATION OF BIOLOGICAL CELL MEMBRANES

In the late 1960s, Sale found that the action of short electric pulses upon biological cell membranes made them transiently permeable without damaging the membrane structures.[1] When he applied high electric fields up to 25 kV/cm to suspensions of the cells, the potential difference across the membrane of the spherical cell increased. This phenomenon caused conformational changes to occur in the membrane structure, resulting in the loss of its semipermeable properties and lysis of the cells.[2]

Another researcher later found that electric impulses cause transient permeability changes in the membranes of vesicles storing biogenic amines which may lead to stimulated neuro-humoral secretion.[3] Studies of cell membranes in Escherichia coli B163 and B525 demonstrated dielectric breakdown using a Coulter counter, with hydrodynamic jet currents focusing close to the orifice of the counter. By plotting the relative pulse height for compensated amplification of a certain size of the cells against increasing detector current, the transcellular ion flow showed differences for the potassium-deficient mutant B525 in comparison with the wild-type B163, indicating a change in the membrane structure of B525.[4]

Researchers also observed that red blood cells began to lose haemoglobin after exposure to a pulsed electric field when the strength of the field exceeded a certain threshold. The haemoglobin fraction found in the supernatant was designed against the outer electric field strength, and resulted in the classic sigmoid curve characterizing the dielectric decomposition of mammalian cell membranes. They discovered that very short (<1ms), high voltage pulses can dramatically increase the permeability of cell membranes.[5]

Reversible electrical degradation of cell membranes is the fundamental cause of electro-permeabilization (also referred to as electro-injection or electroporation). Local degradation of the cell membrane occurs when the membrane potential exceeds 1V at room temperature and 2V at 4°C. The

breakdown voltage depends on the duration of the pulse. A short pulse (<1 μs) causes transient decomposition at 1V, while a pulse of a longer duration than 10ms causes transient decomposition at 0.5 V. At pulses over 50-100 μs, degradation causes irreversible cellular membrane destruction, leading to cell death.[5,6,7,8,9,10]

1.2 ELECTROFUSION OF BIOLOGICAL CELLS

Exposure of the microorganism *Dictyostelium discoidea* to short (40ps) electrical pulses with amplitudes between 4 and 6 kV/cm resulted in the cells melting together, forming larger cells containing more than 40 nuclei of cells evenly as they lyze.[11]

Red blood cells exposed to an electrically flawed AC with an amplitude of 2.5kV/cm and frequencies between 0.5 and 2 MHz were found to form chains of cells between the electrodes which remain after exposure, and which might lead to a possibility of cell fusion.[12] Zimmerman and colleagues applied this phenomenon to the inclusion of cells of various origins utilizing electrical degradation of cell membranes. This experiment resulted in hybrid cells that joined the properties of two varied cells into a single cell.[13,14,15,16,17,18,19]

The fusion rate of yeast cells treated with polyethylene glycol and Ca^{2+} ions and exposed to a high electric field (2.5 - 5 kV.cm-1) increased by a factor of about 200 for different strains of yeast saccharomyces cerevisiae, as opposed to fusion without electric field exposure. This method enables the production of a large number of cell hybrids for various purposes.[20,21]

1.3 ELECTRO-PULSE ENHANCED DNA TRANSFECTION

Compared to biochemical techniques, electric field mediated transfer of DNA into cells is beneficial, and simple and easy to apply for DNA transfection.[22] When exposing plasmid DNA containing the herpes simplex thymidine kinase (TK) gene and mouse L-cells lacking the TK gene, to electrical fields (8 kV/cm,5μs), a strong expression of TK-DNA was found in the mouse cells.[23] A possible model for the transfer of DNA-plasmids into cells by electroporation is an electro diffusive migration of DNA through the electroporated membrane.[24]

The effects of exposure of biological cells to high-voltage electric pulses have so far been explored in terms of electrofusion used for the production of cell hybrids and monoclonal antibody production, or electroporation induced DNA transport across the cell membrane.[11,25,22,23 5521]

1.4 ELECTRO-PULSE ENHANCED CHEMO-THERAPY *EpEChT*

Okino and Mohri (1987) first reported the possibility of using the electroporation effect for enhanced transport of the anticancer drug across the cell membrane.[26] They treated hepatocellular carcinoma (AH-109A) in rats through an administration of 5 mg of Bleomycin intra-muscularly and exposed the tumours to an electric pulse with an amplitude of 5 kV/cm and a duration of 2 ms. Four days after the treatment, the tumour size had decreased significantly, while tumours treated with electric impulses or Bleomycin alone showed no decrease in growth. Okino and Mohri suggested that electro-enhanced chemotherapy should produce better results than chemotherapy alone.[26,27]

1.5 CLINICAL ELECTRO ENHANCED CHEMO-THERAPY (*EpEChT*)

The first clinical trial with *EpECT* was performed on head and neck tumours by Mir and colleagues in France in 1991.[28] In 1998 the Swedish Radiologist Dr Nordenström at the Karolinska Institute in Stockholm, developed electrochemical therapy (EChT) as a minimally invasive electrotherapeutic technique, using direct current for the treatment of cancer and haemangioma tumours.[29]

Application of electric pulses in vivo used to augment the chemotherapeutic efficiency in cancer treatment has commonly been referred to as electro-chemotherapy and is abbreviated as ECT. However, this abbreviation is also associated with electro-convulsive therapy, as used in psychiatry for the treatment of depression. In this book, *EpECT* is used as an abbreviation for *electro-pulse-enhanced cancer therapy*, while electro-pulse enhanced chemotherapy is abbreviated as *EpEChT*.[30] This latter is a mode of *EpECT* tumour treatment that has been employed mostly for subcutaneous and cutaneous malignancies.[31,32] However, it is also employed to treat soft-tissue sarcomas[33], glioma in the brain[34], liver tumours [35,36,37] and tumours in the pancreas,[38] and has shown auspicious results.

In electro-pulse enhanced chemotherapy treatment, only cells exposed to strong electric fields respond immediately. The permeabilized tumour cells become much more accessible to hydrophilic molecules since the lipophilic membrane barrier generally rejects these. Bleomycin, a very toxic anticancer agent, has proven to be a particularly efficient drug in *EpEChT*, and is by far the most often used, but Cisplatin, another anticancer agent, has also been found to be effective.

Chapter II reviews the electrochemical and biophysical principles of pore formation. In the clinical use of electro-pulse-enhanced chemotherapy, it is important to control the exposure of the patient to an electric field.

Chapter III discusses electro-dosimetry, the measurements of the absorbed energy in a tumour through the electric pulse treatment.

In Chapter IV, radioactive tracers and gamma-camera measurements are employed to explore the effect of the enhanced accumulation of administered drugs in the treatment area.

In Chapter V, various preclinical studies of electro-enhanced cancer therapy are reviewed, as are trials using both Bleomycin, Cis-Platin and other drugs, as well as their combinations.

In chapter VI clinical studies of electro-enhanced cancer therapy using various devices are reviewed, considering both Bleomycin, Cis-Platin, other drugs, and their combinations.

The safety aspects of performing clinical electro-enhanced cancer therapy are discussed in chapter VII. The closing chapters then detail new dimensions of EpECT to summarize the clinical potential in applying electro-pulse treatment to enhance the effect of established cancer therapy regimes.

CHAPTER II

BIOPHYSICAL PRINCIPLES OF *EPECT*

2.1 CELL MEMBRANE

Cell membranes with a thickness of about 7 to 10 nano-m enclose all single cells in the body. The cell membrane is made up of a double layer of phosphorous glycerides, consisting of phosphoric acid and fatty acids (see Figure 2-1a). The end of these molecules with the hydrophilic phosphoric acid (which attracts water) cover each side of the cell membrane, while the other ends of long-chain hydrocarbon chains that are hydrophobic (water repellent) fill the space between the hydrophilic surface layers (see Figure 2-1b).

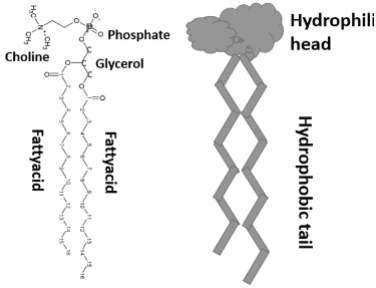

Figure 2-1a
Phosphorous glycerides consisting of phosphoric acid and fatty acids

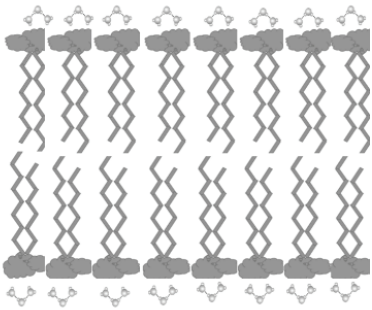

Figure 2-1b
The hydrophilic phosphoric acid (which attracts water) covers each side of the cell membrane, while the other end of long fatty acid hydrocarbon chains that are hydrophobic (water repellent) fills the space between the surface layers.

This structure of the cell membrane creates an electrically insulating double layer of phosphorous glycerides. For the cell to communicate with the environment, there are a large number of various proteins embedded in

the cell membrane. A group of these proteins consists of channel proteins transporting distinct ions through their channels. The difference in concentrations of positive sodium, potassium, calcium ions and negative chloride ions on either side creates a membrane potential that is the basis for the emergence of bioelectric phenomena.

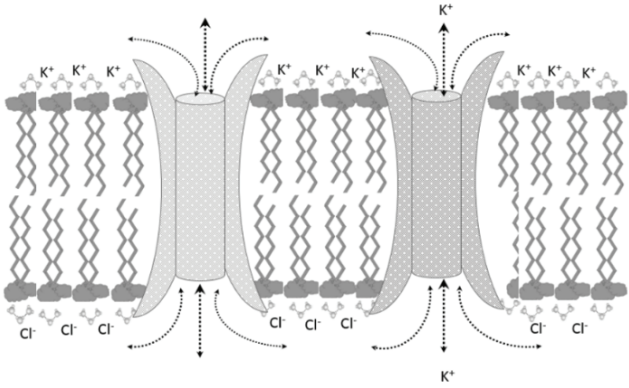

Figure. 2-2
The structure of a cell membrane consists of two lipid layers, with hydrophilic phosphate groups on the surface and hydrophobic fatty acid tails which fill the space inside the membrane. The channel protein embedded in the membrane transports sodium, potassium, calcium and chloride molecules through the membrane. This structure, with differences in ion concentrations on either side of the membrane, generates the membrane potential and the appearance of bioelectric phenomena.

2.2 MEMBRANE POTENTIAL

Differences in the concentrations of ions on opposite sides of a cellular membrane create an electrical potential called the Nernst potential (U_{ion}), determined by the charge on the ion q_{ion} and the ion-concentrations on the inside and outside of the cell.

$$U_{ion} = \frac{R \cdot T}{q_{ion}} \ln \frac{\{ion\ concentration\ outside\ the\ cell\}}{\{ion\ concentration\ inside\ the\ cell\}} \qquad \text{Eq. 2.1}$$

Sodium (Na^+) and chloride (Cl^-) ions appear in high concentrations on the extracellular side of the membrane and in low concentrations on the intracellular side. The concentration of potassium in normal rest conditions is usually about 30-50 times higher inside the cell compared to outside. Potassium ions, therefore, strive to flow out of the cell and accumulate on the outside of the membrane. As the negative charged q^- ions remain in the cell (mainly chloride), accumulating on the inside of the membrane, an electric field directed into the cell develops through the outflow of positively q^+ charged potassium. This condition continues until the force created by the electric field ($\vec{F} = \vec{E} \cdot q^+$), which is directed inwards, inhibits the outbound transport of potassium ions. The electric potential, Φ, is the amount of work needed to move a positive unit charge, $q^+ = 1$, a distance $\Delta\ell$ inside the electric field E without producing any acceleration. The work of moving the K^+ ions a distance $\Delta\ell$ in the E field inside the membrane is thus:

$$\vec{F} \cdot \Delta\ell = \vec{E} \cdot \Delta\ell \cdot q^+ = \Delta\Phi \qquad \text{Eq. 2.2}$$

The potential energy $\Delta\Phi$ is equal to the difference between the membrane potential Φ_M and the Nernst potential for the potassium ion Φ_K , $\Delta\Phi = \Phi_M - \Phi_K$. At equilibrium, the potential energy is zero, $\Delta\Phi = 0$ and $\Phi_M = \Phi_K$.

The general Nernst equation for an arbitrary ion κ is:

$$\Phi_i - \Phi_e = \Phi_\kappa = -\frac{R \cdot T}{q_\kappa \cdot F} \cdot ln \frac{C_I^\kappa}{C_{E,\kappa}}; \ [V] \qquad \text{Eq. 2.3}$$

where

Φi = potential on the inner side of the membrane;
ΦE = potential on the external side of the membrane;
Φ_κ = equilibrium potential for the κ^{th} ion across the membrane $\Phi_i - \Phi_E$ i.e., the Nernst potential U_κ [V];
R = gas constant [8.314 J/(mole·degree Kelvin)];
T = absolute temperature [degree Kelvin];
q_κ = valence of the kth ion;
F = Faraday's constant [$9.649 \cdot 10^4$ As/mole];
C_I^κ = intracellular concentration of the κ^{th} ion;
C_E^κ = extracellular concentration of the κ^{th} ion.

The potential that occurs is called the membrane potential, and is in the order of -40 mV to -70 mV. By introducing a temperature of 37 ° C, which corresponds to T = 273 + 37 °Kelvin, q=+1 for the valence of the potassium ion, and replacing the natural logarithm (ln) with the tenth logarithm ($\log_{10}$), the equation for a monovalent cation (q=+1) can be written as:

$$\Phi = -61 \cdot log_{10} \frac{C_I}{C_E} \ [mV] \qquad \text{Eq. 2.4}$$

- The equilibrium's potential for potassium(q=+1) Φ_K is -88 mV at an intra-cellular potassium concentration of 140mM and an extracellular concentration of 5 mM.
- The equilibrium's potential for sodium(q=+1), Φ_{Na}, is +61 mV at an intra-cellular sodium ion concentration of 14mM and an extracellular concentration of 140 mM.
- The equilibrium's potential for chloride(q=-1), Φ_{Cl}, is -69 mV at an intra-cellular chloride ion concentration of 8mM and an extracellular concentration of 110 mM.
- The equilibrium's potential for calcium(q=+2) Φ_{Ca}, is +140 mV at an intra-cellular calcium ion concentration of 0.1 μM and an extra-cellular concentration of 4mM

The interactions that generate the total rest potential are modelled by the Goldman equation, which is similar to the Nernst equation shown above. The Goldman equation considers the charges of the current ions as well as the difference between their internal and external concentrations. However, it also takes into account every κ^{th} ion's relative permeability $P\kappa$ of the plasma membrane.

$$\Phi_M = \frac{R \cdot T}{F} \cdot ln\left(\frac{P_K \cdot C_E^K + P_{Na} \cdot C_E^{Na} + P_{Cl} \cdot C_I^{Cl}}{P_K \cdot C_I^K + P_{Na} \cdot C_I^{Na} + P_{Cl} \cdot C_E^{Cl}}\right) \qquad \text{Eq. 2.5}$$

In excitatory cells (nerve and muscle cells), the membrane potential is usually around -60 mV.

2.3 MEMBRANE EXPOSED TO ELECTRIC DC FIELD

The electric field E is written as the derivate of the electric potential, Φ,

$E = -\frac{d\Phi}{dr}$ in vector form $\bar{E} = grad\Phi = \nabla\Phi$ Eq. 2.6

Then, by Coulomb's and Gauss's laws, we get Poisson's equation

$E = \frac{1}{4\pi \cdot \varepsilon_o}\frac{q}{r^2} = -\frac{d\Phi}{dr}$; $\frac{dE}{dr} = -\frac{d^2\Phi}{dr^2} \approx \frac{\rho}{\varepsilon_o}$; Eq. 2.7

Alternatively, in vector sign $-\nabla^2\Phi = \frac{\rho}{\varepsilon_o}$; Eq. 2.8

where:

ρ is the charge density (including bound charge)

ε_o is the permittivity = 8.854187817...×10^{-12} F·m^{-1} (farads per metre).

When applying an external electric field, the superposed membrane potential is related to the charge density by Poisson's equation (as in Eq. 2.8). Where no charges are involved, solutions of the Laplace equation (Eq. 2.9) give the electric field in the extracellular and intracellular space.

$$\nabla^2\Phi = 0 \qquad \text{Eq. 2.9}$$

We assume that the model of a spherical cell according to Figure 2-3 has an inner radius **a** and an outer radius **b** with the membrane thickness **d**. The conductivity of the cell's internal material (cytoplasm) is $\boldsymbol{\sigma_I}$, the cell membrane $\boldsymbol{\sigma_M}$ and the extracellular fluid $\boldsymbol{\sigma_E}$.[5] The electrical potential in the corresponding areas is assumed to be Φ_I, Φ_M, and Φ_E.

Figure 2-3 shows a model of a cell as a layered lossless dielectric sphere subjected to a uniform electric field $\vec{E} = E \cdot \vec{x}$. The concentric outer shell and inner core have conductivities σ_I and σ_M respectively, and the outer radius is b and the inner radius a. The solutions to the Laplace equation (2.9) for the electric potentials in the three regions I, M and E are assumed to be as follows:

$$\Phi_E(\mathrm{r},\theta) = \left(-E \cdot r + \frac{B}{r^2}\right) \cdot \cos\theta\,; \text{ for r > b} \qquad \text{Eq.2.10a}$$

$$\Phi_M(\mathrm{r},\theta) = \left(-B \cdot r + \frac{C}{r^2}\right) \cdot \cos\theta\,; \text{ for b > r > a} \qquad \text{Eq.2.10b}$$

$$\Phi_I(\mathrm{r},\theta) = -D \cdot r \cdot \cos\theta\,; \text{ for r < a} \qquad \text{Eq.2.10c}$$

where

$\Phi_I = 0$, $\Phi_M = A \cdot r \cdot \cos\theta$, and $\Phi_E = \frac{B}{r^2} \cdot \cos\theta$

The border conditions for the two interfaces r =a and r =b are as follows:

$$\Phi_I(a) = \Phi_M(a) \text{ and } \left(\sigma_I/a\right)\left(d\Phi_I/dr\right) = \left(\sigma_M/a\right)\left(d\Phi_M/dr\right) \qquad \text{Eq. 2.11a}$$

$$\Phi_M(b) = \Phi_E(b) \text{ and } \left(\sigma_M/b\right)\left(d\Phi_M/dr\right) = \left(\sigma_E/b\right)\left(d\Phi_E/dr\right) \qquad \text{Eq. 2.11b}$$

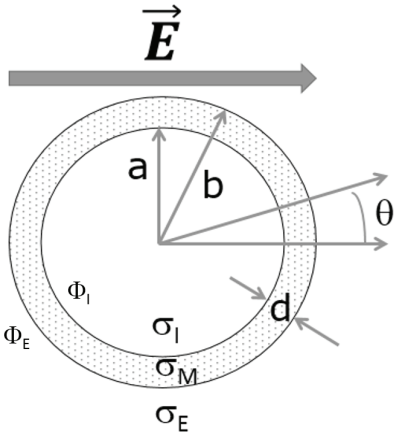

Figure 2-3
An applied external electric field $\vec{E}$ on a spherical cell with the inner radius **a,** and the outer radius **b** with the membrane thickness **d**. The conductivity of the cell's internal material (cytoplasm) is $\boldsymbol{\sigma_I}$, the cell membrane $\boldsymbol{\sigma_M}$ and the extracellular fluid $\boldsymbol{\sigma_E}$.[5]

With these assumptions, the conductivity for the cytoplasm 'σ_I' is about the same as the extracellular space 'i.e.≈ σ_E', and is much larger than the cell membrane 'i.e. >> σ_M'. The Laplace equation gives the following expression for the part of the potential difference $\Delta\Phi_M$ across the membrane depending upon the externally applied electric field E_0 .[5]

$$\Delta\Phi_M = 1.5 \cdot b \cdot E_0 \cdot \cos\theta \qquad \text{Eq.2.12}$$

If, for non-spherical cells, `$1.5 \cdot \cos\theta$´, is replaced by a form factor f, the equation for $\Delta\Phi_M$ becomes:

$$\Delta\Phi_M = f \cdot b \cdot E_0 \qquad \text{Eq.2.13}$$

For a cell with the shape of an ellipsoid of length L and width B, the form factor f becomes f = L / (L - 0.33·B).[5] At an externally applied field strength of 4400 V/cm, which corresponds to a membrane potential of 1.5 V in bovine red blood cells with an average radius of 2.2 μm, a dielectric degradation of the membrane occurred, which caused a 50% leakage of haemoglobin.[5] Cancer cells, which may be 10 times larger in diameter, can achieve the conditions for a dielectric breakdown at a membrane potential of 1.5 V, already around 400 V/cm.[39,40]

2.4 ELECTRIC PULSE PARAMETERS AND FREQUENCY

2.4.1 ELECTROPORATION WITH PULSED AC FIELDS

When an AC-field is applied to cell membranes, the induced trans-membrane potential becomes strongly dependent upon the frequency of the applied field; when the frequency approaches the relaxation time 'τ' of the

membrane. Schwan (1983) derived a relationship from the basic electromagnetic theory for the calculation of the trans-membrane potential '$\Delta\psi_{membrane}$' induced by an oscillating electric field '$E_{applied}$'.[41]

$$\Delta\psi_{membrane} = \frac{1.5 \cdot r \cdot E_{applied} \cdot \cos\theta}{\sqrt{1 + (\omega \cdot \tau)^2}} \quad \text{Eq.2.14}$$

$$\tau = r \cdot C_{membrane} \cdot (\rho_{internal} + \rho_{external}/2) \quad \text{Eq.2.15}$$

where

ω	ω=2πf; Angular frequency (rad.s^{-1})
f	Frequency (Hz)
$C_{membrane}$	Specific capacitance of the membrane (F.m^{-2})
$\rho_{internal}$	Resistivity of the internal medium (Ω.m)
$\rho_{external}$	Resistivity of the external medium (Ω.m)
$E_{applied}$	$=E^0_{applied} \cdot \sin(\omega t)$ Applied field strength V.m^{-1}
t	Time(s)
r	Radius of the cell

The maximum membrane potential induced by an applied AC field is:

$$\Delta\psi^{max}_{membrane} = \frac{1.5 \cdot r \cdot E^0_{applied}}{\sqrt{1 + (\omega \cdot \tau)^2}} \quad \text{Eq. 2.16}$$

$$\Delta\psi^{max}_{membrane} = \frac{1.5 \cdot r \cdot E^0_{applied}}{\sqrt{1 + (2\pi f \cdot \tau)^2}} \quad \text{Eq. 2.17}$$

The concept of electroporation using reactively-coupled AC-fields of kilohertz frequencies is attractive, because it may enable the use of non-invasive electrodes capable of producing electric fields of sufficient strength and depth in tissue to enhance cellular uptake of drugs, thus avoiding the need for invasive needle electrodes, and also reducing the likelihood of painful neural stimulation.

Under physiological conditions, a typical value of the capacitance of the cell membrane is in the order of $C_m = 10^{-2}$ F.m^{-2}. The values of the resistivity values $\rho_{internal,}\ \rho_{external}$ are in the order of 1 Ω.m. The typical radius `r´ of human cells are in the order of r = 10^{-6} -10^{-4} m. Thus, the frequency limits of the low-frequency potential ranges are in the order of 10^2-10^3 kHz.

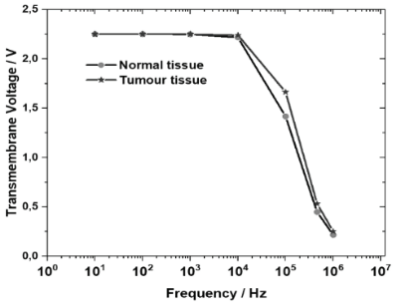

Figure 2-4a
Theoretical calculation of electric-field induced trans-membrane potential, given an applied field strength of 1000 V/cm.

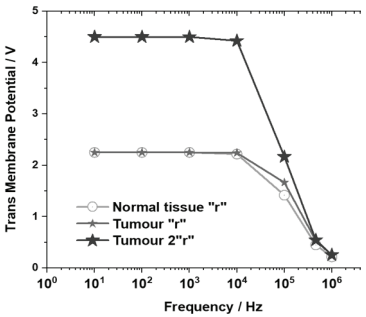

Figure 2-4b
Theoretical calculation of electric-field induced trans-membrane potential, given an applied field strength of 1000 V/cm.

Grosse and Schwan (1992) presented a model for the calculation of cellular trans-membrane potential in the case of very weak electrolytes $\rho >> 1$ Ω.m.[42] This model includes the effect of a variety of factors on transmembrane potential (see Figure 2-3), including medium conductivity and permittivity, cell membrane conductance and permittivity, surface admittance, and space charge effects. Measurements of the *ex vivo* electrical conductivity of a normal human liver and metastatic tumour tissue showed that the conductivity of tumour tissue was significantly higher over the entire frequency range (10 Hz to 1 MHz), with more pronounced differences at lower frequencies.[43]

The Foundation for Research on Information Technologies in Society (ITIS) has an excellent database for the dielectric properties of various human tissues:

https://www.itis.ethz.ch/virtual-population/tissue-properties/database/dielectric-properties/

In Table 2-1, the conductivity (S/m) and resistivity (Ω.m) are given for muscle tissue derived from the ITIS database. According to reported experimental results, the values for tumours are predicted to differ from those for muscles by a factor of about 3 in the frequency range of 10-200 kHz.[44,43] Fitting actual ratios to frequency according to the data of Haemmerich et al. (2009) results in the following Gauss equation:

$$\frac{\sigma_{Tum}}{\sigma_{NT}} = 5.66756 - \frac{10.19901}{2.1077*\sqrt{\pi/2}} * e^{-2*\left[\frac{log_{10}(f) - 5.73715}{2.1077}\right]^2} \quad \text{Eq. 2.18}$$

where:

σ_{Tum}	= Conductivity of tumour tissue;	S/m
σ_{NT}	= Conductivity of normal muscle tissue;	S/m
f	= Frequency;	Hz

In Table 2-1, the conductivity values for muscle tissue are derived from the ITIS database, and estimated values for tumour tissue are provided by applying the equation Eq. 2.18 to the muscle tissue data.

Table 2-1
The conductivity (S/m) and resistivity (Ω.m) for muscle and tumour tissue.

Frequency kHz	Muscle S/m	Tumour S/m	Muscle Ω.m	Tumour Ω.m
10	0.341	1.594	2.933	0.627
20	0.345	1.429	2.899	0.700
40	0.350	1.227	2.857	0.815
80	0.358	1.018	2.793	0.982
100	0.362	0.957	2.762	1.045
110	0.364	0.933	2.747	1.072
120	0.366	0.911	2.732	1.097
140	0.370	0.876	2.703	1.141
180	0.379	0.830	2.639	1.205
200	0.384	0.815	2.604	1.226

The equations for normal muscle tissue conductivity variation with frequency fitted to the data of the ITIS database in the frequency f interval 1 - 4000 Hz are as follows:

$$\sigma_m = 0.2 + 0.00003 \cdot f \text{ S/m} \quad \text{Eq.2.19}$$

In the frequency f interval 4000 – 200 000 Hz:

$$\sigma_m = 0.337 + 3.49 \cdot 10^{-7} \cdot f - 1.45 \cdot 10^{-12} \cdot f^2 + 4.49 \cdot 10^{-18} \cdot f^3 \text{ S/m} \quad \text{Eq.2.20}$$

In Figure 2-4a, calculations are based on Eq. 2.17 with data for normal tissue cellular radius r = 15 μm, and $C_m = 0.001$ F.m^{-2}; $\rho_{in} = 10$ Ω.m; $\rho_{ex} = 15$ Ω.m. For tumour cellular radius r = 15 μm, and $C_m = 0.001$ F.m^{-2}; $\rho_{in} = 3.3$ Ω.m; $\rho_{ex} = 15$ Ω.m.[43]

In Figure 2-4b, calculations are based on Eq. 2.17 with normal tissue data for cellular radius r = 15 μm.
Cm = 0.001 F.m^{-2}, ρ_{in} = 3.5-13 Ω.m, ρ_{ex} =15 Ω.m, and with tumour tissue data for cellular radius r = 15 μm or 30 μm, C_m = 0.001 F.m^{-2}, ρ_{in} = 1.9-2.4 Ω.m, and ρ_{ex} =15 Ω.m. [43]

In agreement with experimental findings, the theoretical model also predicts that a trans-membrane voltage of sufficient magnitude to cause electroporation appears at an applied field strength of 1200 V/cm at 20 kHz.[45]

A study by Chen et al. (2008) showed that 20–160 kHz in electric fields of peak amplitude 700–2000 V/cm, when applied as a series of short pulses, induce electroporation in cell membrane.[46] Increasing the field strength above 1100 V/cm results in higher cell electroporation efficiency, but increases cell death. In the study, total electroporation yield is only weakly dependent on the frequencies in the frequency range used, although the results suggest a peak of Calcein uptake at 60 kHz. For the parameter values chosen, AC and DC electroporation produced similar yields. Marszalek et al. (1990) studied electroporation in a murine myeloma cell line using AC fields, in the frequency range 100–300 kHz and with field strengths between 500 V/cm and 1500 V/cm.[45]

The study identified a threshold electric field around 600 V/cm using a single pulse of 200ms. However, the article gives no quantitative information about electroporation efficiency and cell viability. The threshold field strength for electroporation in the study of Marszalek et al. (1990) was approximately half of that found through the study by Chen et al. (2008).[45,46]

This result may be due to several factors, including differences in the conductivity of the extracellular medium, pulsing regime, the geometry of the electrodes, the chemistry used to detect electroporation, and cell lines.

The results of Chen et al. (2008) offer conclusive evidence of electroporation using pulsed with AC fields beyond the kilohertz range. The results also suggest an optimal frequency range of 40–120 kHz at a field strength of 1700 V/cm and total exposure time 40ms.[46]

Influenced by these findings of an optimal frequency window for electroporation achievements, the author analysed the frequency content of the various pulse sequences usually used in the protocols for electro-enhanced chemotherapy.

A recent investigation of electroporation with 5 ms bursts of sinusoidal signals in the frequency range of 8 -130 kHz showed that permeabilization appeared at about 100% up to 50 kHz, and then drastically decreased to

about zero above 130 kHz.[47] This result is in solid agreement with the results of the theoretical calculations displayed in Figure 2-4.

2.4.2 FOURIER ANALYSIS OF *EpECT* PULSES

The **Fourier analysis** decomposes a pulse V(t) in the time domain, into the frequencies f that make up a frequency distribution G(f).

A. A SINGLE RECTANGULAR PULSE

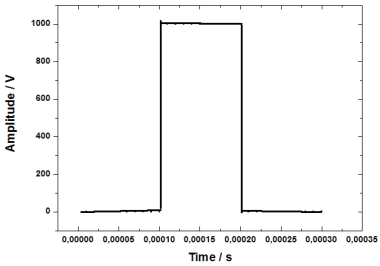

Figure 2-5a
Single rectangular pulse of 1000 V amplitude, and 100µs pulse-length

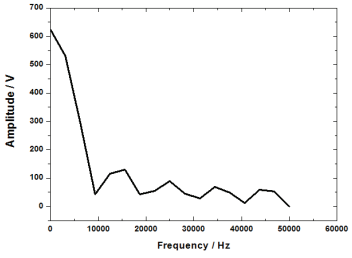

Figure 2-5b
The positive frequency content of the single rectangular pulse in Figure 2-5a

The result of the Fourier analysis of a single rectangular pulse according to the ESOPE protocol displayed in Figure 2-5b shows that the main content of frequencies is in the low energy range < 1 kHz. In the region of optimal electroporation (30- 120 kHz), there is only a minor content of frequencies.

B. A PULSE-TRAIN OF RECTANGULAR PULSES

The result of the Fourier analysis of a pulse train of a rectangular pulse according to the ESOPE protocol displayed in Figure 2-6b shows that there is a peak at 5 kHz corresponding to the 200µs wavelength. There is, however, still a large amplitude at low frequencies, which causes nerve excitation and pain, which this study attempted to avoid by using a 5 kHz pulse-train. In the region of optimal electroporation 30 - 120 kHz, there is still only a minor content of frequencies.

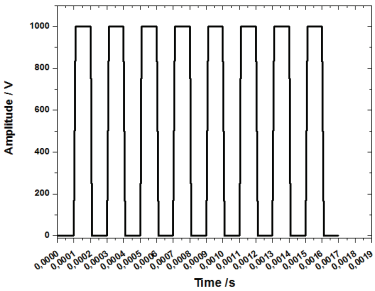

Figure 2-6a
Pulse-train of 8 rectangular pulses of 1000 V amplitude. 100μs pulse-length and 200 μs wave-length (5 kHz).

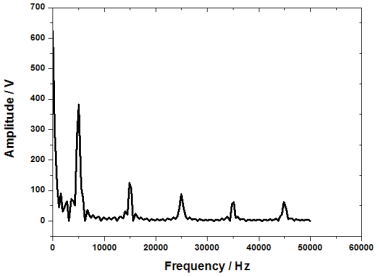

Figure 2-6b
The positive frequency content of the pulse-train of 8 rectangular pulses in Figure 2-6a

The conductivity for healthy muscle tissue averaged over the positive frequency spectrum of Figure 2-6b for each pulse of the pulse train becomes 0.30 S/m. The specific energy thus delivered to a pulse train of 8 rectangular pulses of 1000 V amplitude and 100μs pulse-length is 2208 J/kg. By applying Eq.2.17, the conductivity of tumour tissue conductivity was estimated to be 1.68 S/m, and the specific energy delivered to a pulse train was put at 12514 J/kg.

C. A SINGLE EXPONENTIALLY DECREASING PULSE

Figure 2-7b displays an exponentially decreasing pulse of 139 μs half-time, 1000 V maximum amplitude, and 100μs pulse-length. The result of the Fourier analysis in Figure 2-7b shows that the main content of frequencies is in a broad peak around 7 kHz, with a substantial contribution in the region of optimal electroporation 30 - 120 kHz.

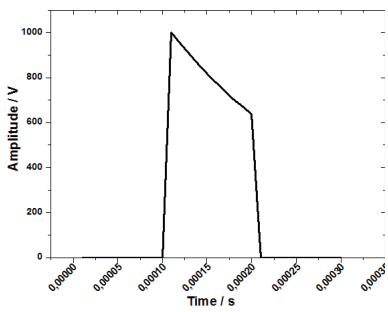

Figure 2-7a
An exponentially decreasing pulse of 139 μs half-time, 1000 V maximum amplitude, and 100μs pulse-length.

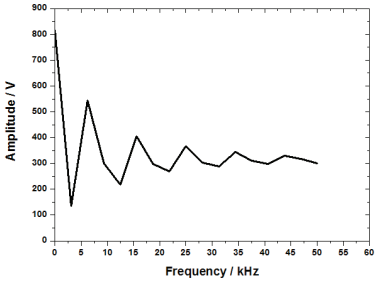

Figure 2-7b
The positive frequency content of the single exponentially decreasing pulse in Figure 2-7a.

D. A PULSE TRAIN OF EXPONENTIALLY DECREASING PULSE

Figure 2-8a displays a pulse-train of 8 exponentially decreasing pulses of 139 μs half-time, 1000 V maximum amplitude, and 100 μs pulse-length. The result of the Fourier analysis displayed in Figure 2-8b shows that the main content of frequencies is in a peak around 5 kHz corresponding to the pulse repetition frequency, and that there are several peaks with a minor contribution in the region of optimal electroporation 30- 120 kHz.

The conductivity for healthy muscle tissue averaged over the positive frequency spectrum of Figure 2-8b for each pulse of the pulse train becomes 0.34 S/m. The energy thus delivered to a pulse train of 8 exponentially decreasing pulses of 139 μs half-time, 1000 V maximum amplitude, and 100 μs pulse-length is 2030 J/kg. By applying Eq.2.17, the conductivity of tumour tissue conductivity was estimated at 1.94 S/m and the specific energy delivered to a pulse train was put at 11 500 J/kg.

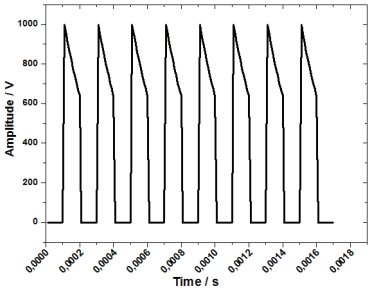

Figure 2-8a
Pulse train of 8 exponentially decreasing pulses of 139 μs half-time, 1000 V maximum amplitude and 100μs pulse-length.

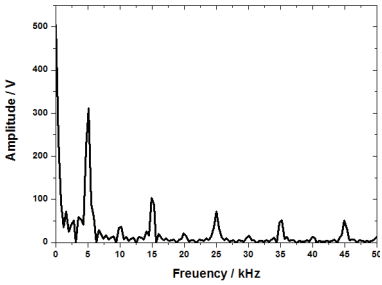

Figure 2-8b
The positive frequency content of the exponentially decreasing pulse in Figure 2-8a.

E. SINGLE SINUSOIDAL PULSE AT 10 kHz

Figure 2-9a displays a single sinusoidal pulse of ±1000 V max amplitude and 9677.5 Hz frequency corresponding to 103 μs pulse-length. The result of the Fourier analysis displayed in Figure 2-9b shows that the main content of frequencies is in a peak around 10 kHz, with a minor contribution of overtones at a higher frequency.

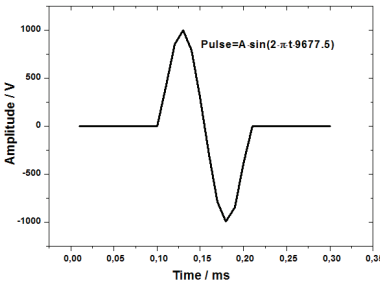

Figure 2-9a
A sinusoidal pulse of ±1000 V max amplitude and 9677.5 Hz frequency corresponding to 103 μs pulse-length.

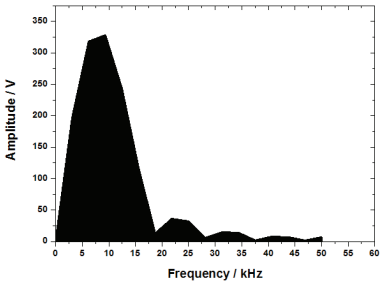

Figure 2-9b
The positive frequency content of the sinusoidal pulse in Figure 2-9a.

F. PULSE TRAIN OF 10 KHZ SINUSOIDAL PULSES

Figure 2-10a displays a pulse train of 8 sinusoidal pulses of ±1000 V max amplitude and 9677.5 Hz frequency corresponding to 103 μs pulse-length. The result of the Fourier analysis displayed in Figure 2-10b shows that the main content of frequencies is in one peak around 5 kHz corresponding to the pulse train frequency and another peak at 10 kHz corresponding to the frequency content of the single pulses. There is also a third peak corresponding to the overtone of the sinusoidal pulse. There are minor contributions in the regions of low frequencies <1 kHz and of optimal electroporation 30 - 120 kHz.

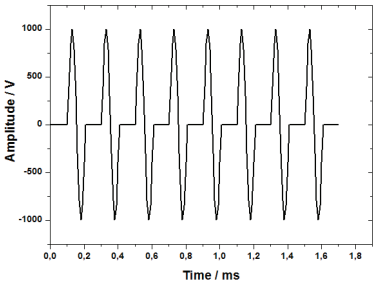

Figure 2-10a
Pulse-train of 8 sinusoidal pulses of ±1000 V max amplitude and 9677.5 Hz frequency corresponding to 100 μs pulse-length.

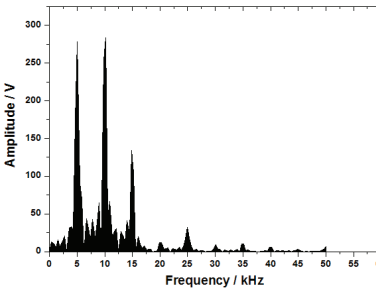

Figure 2-10b
The positive frequency content of the pulse-train of 8 sinusoidal pulses in Figure 2-10a.

The conductivity for healthy muscle tissue averaged over the positive frequency spectrum of Figure 2-10b for each pulse of the pulse train becomes 0.34 S/m. The energy thus delivered to a pulse train of 8 sinusoidal pulses of ±1000 V max amplitude and 9677.5 Hz frequency corresponding to 100 μs pulse-length is 1409 J/kg. Thus, this pulse train must have 14 pulses to deliver the same energy as the ESOP protocol of 8 rectangular pulses of 100 μs at 5 kHz.

2.5 ELECTRO-PORES CREATION AND EVOLUTION FOR DRUG DELIVERY

The application of external electrical fields to biological cells creates initially hydrophobic pores at a rate depending on the absorbed energy.[48,49,50] Smaller pores of radius r < 0.5 nm are rapidly sealed, while

larger ones are stable hydrophilic pores with a radius > 0.8 nm.[51] The rate of formation of such stable pores is modelled by the following equation.[51, 52]

$$\frac{dN}{dt} = \alpha \cdot e^{(\Phi_M/\Phi_{Ep})^2} \cdot \left(1 - \frac{N}{N_{eq}(\Phi_M)}\right) \qquad \text{Eq. 2.21}$$

where

N = Pore density (m^{-2})

α = Creation rate coefficient ($1 \cdot 10^9$ $m^{-2}.s^{-1}$)

ϕ_M = Membrane potential

ϕ_{Ep} = Threshold potential for electroporation ≈258 mV

$N_{eq}(\phi_M)$ = Pore density at membrane potential ϕ_M given by Eq. 2.22

N_0 = $1.5 \cdot 10^9$ (m^{-2}).[51]

$$N_{eq}(\Phi_M) = N_o \cdot e^{2.46 \cdot (\Phi_M/\Phi_{Ep})^2} \qquad \text{Eq. 2.22}$$

The bilayer energy W_M of a membrane with K electro-pores of an initial radius r_j depends on the following parameters: [53]

1. Steric repulsion of lipid heads $\sum_{j=1}^{K} \beta \cdot \left(\frac{r_s}{r_j}\right)^4$;
2. Edge energy of the pore perimeter $\sum_{j=1}^{K} 2\pi\gamma r_j$;
3. The effect of the pores on the membrane tension ; where $\sum_{j=1}^{K} -\pi \cdot \sigma_{eff}(A_p) \cdot r_j^2$; $\sigma_{eff}(A_p)$ is a function of the combined area of pores $A_p = \sum_{j=1}^{K} \pi r_j^2$;
4. Contribution to the membrane potential $\sum_{j=1}^{K} \int_0^{r_i} F(r_j \Phi_M) \cdot dr$.

As the electro-pores expand their total area and ($A_p = \sum_{j=1}^{K} \pi r_j^2$) increases, the effective membrane tension σ_{eff} felt by each pore decreases according to the equation:[54]

$$\sigma_{eff} = 2 \cdot \sigma' - \frac{2 \cdot \sigma' - \sigma_o}{(1 - A_p/A_M)^2} \qquad \text{Eq. 2.23}$$

where

σ' is the specific tension energy (0.02 $J.m^{-2}$) of the hydrocarbon-water;

σ_o is the specific tension energy without pores (0.01$J.m^{-2}$);

A_M is the total area of the cell membrane ($1.26 \cdot 10^{-9}$ m^2 of a cell with a 10 μm radius).

The electric force $F(r.\Phi_M)$ acting on a pore of a toroidal inner surface according to Fig 2-11 is given by Eq. 2.24.[54]

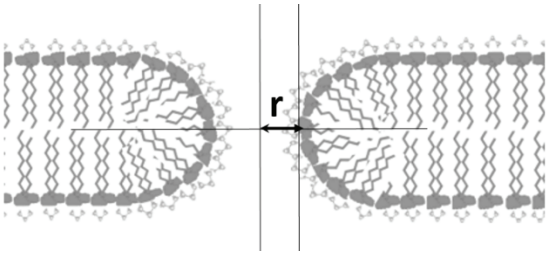

Figure 2-11
Schematic illustration of an electro-pore of radius **r** in the cell membrane

$$F(r.\Phi_M) = \frac{F_{max}}{1 + \frac{0.97 \cdot 10^{-9}}{r + 0.31 \cdot 10^{-9}}} \cdot \Phi_M^2 \qquad \text{Eq. 2.24}$$

where

F_{max} is the maximum of electric force ($0.7 \cdot 10^{-9}$ N.V^{-2} at Φ_M= 1 V).

Thus the energy W of the entire membrane lipid bilayer is:[54]

$$W=\sum_{j=1}^{K}\left\{\beta \cdot \left(\frac{r_s}{r_j}\right)^4 + 2\pi\gamma r_j - \pi \cdot \sigma_{eff}(A_p) \cdot r_j^2 + \int_0^{r_i} F(r_j \Phi_M)\, dr\right\} \qquad \text{Eq. 2.25}$$

The radius of a pore with an initial radius r_j increases in size while the membrane energy is degrading according the following equation:

$$\frac{dr_j}{dt} = -\frac{D}{kT} \cdot \frac{dW_M}{dr_j} \cdot \text{j} = 1,2,,,,,\text{K} \qquad \text{Eq. 2.26}$$

where $\frac{dW_M}{dr_j}$ is the rate of degrading the bilayer energy W_M of a membrane with K expanding electro-pores of an initial radius r_j.

2.6 DRUG DELIVERY THROUGH THE ELECTRO-PORES

The number of pores at the end of the electroporation pulse N_p is estimated by integrating the electro-pore density over the entire cell surface. Data on the effect of field strength from 130 V/cm to 1000 V/cm on a total number of pores distributed over the cell surface have been extracted from the diagrams in *Figure 8* of the work of Krassowska and Filev (2007).[55]

The average pore radius at the end of electroporation is assumed to be r_p, and thus the total cross-section area of electro-pores is $A_p = K \cdot \pi \cdot r_p^2$. As soon as the applied electroporation pulse ends, the created pores start to reseal at a first-order rate with mean decay time τ_p in the order of seconds (Figure 2-12).

$$A_p = K \cdot \pi \cdot r_p^2 \cdot e^{-t/\tau_p} \qquad \text{Eq. 2.27}$$

In an exploration of the enhanced uptake of the radioactive tracer in rats after applied electric pulses of 1000 V/cm field-strength and 100μs pulse-length, the relaxation time τ of the bio-impedance was examined as well.[56] The radioactive tracer ^{99m}Tc-DTPA was administered as a depot intramuscularly (i.m.) in the shoulder of Fischer-344 rats in several fractions of 50 μl each, in 1-minute intervals. Treatment with electric pulses was performed with 2 needle electrodes separated by 8 mm and inserted in the right posterior thigh muscle, through which pulses of 600; 800; 1000;1 200 V/cm field-strength (of 100, 250, and 500 μs pulse-length) were applied. At 6 and 24 hours after electro pulse treatment, a gamma-camera recorded the distribution of ^{99m}Tc-DTPA in the rats.

Before applying the pulse treatment, two measurements of the conductance performed at 2 and 20 kHz through the needle electrodes in the right posterior thigh muscle established the reference level G_{before}. Then, during the treatment, measurements of the conductance took place between each consecutive pulse (Np = 2,4,6,10,12) applied at 1s intervals.

After the last applied pulse, the final conductance G_{after} started to decrease and approach a plateau value. To study the relaxation of the created pores, the conductance measurements were continued 15 seconds after the last pulse at 1s intervals.

The plateau value relative to the conductivity G_{after} that was reached after 15s is a measure of the fraction of reversible electro-permeabilized cells. This value is of importance for the long-term transfer of exogenous substances to the cell and the outflow of immunogenic substances from the cell. Figure 2-12 displays the relaxation curve fitted to the following equation of single exponential decay:

$$G(t) = G_{end}\cdot(f_{irr} + f_{rev}\cdot exp(-t/\tau_p) \qquad \text{Eq.2.28}$$

where

f_{rev} is the fraction of reversible electroporation;
f_{irr} is the fraction of irreversible electroporation;
τ_p is the mean relaxation time.

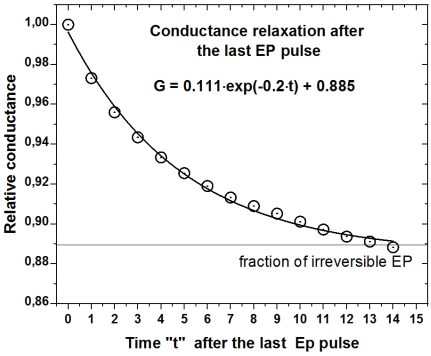

Figure 2-12
Conductance relaxation in seconds after application of 12 pulses with amplitude 1000 V/cm and pulse-length 100 μs in a rat femur muscle.

Figure 2-12 shows that the conductance relaxation with a relaxation rate constant of 0.2 s^{-1} corresponding to a mean relaxation time or mean decay time τ_p for the created pores is 4.8 ± 0.7 s, and the fraction of reversible electro-permeabilized cells is 0.11 (11%).[56]

Directly after electroporation, the drug in the extracellular space has a concentration of $C_{ex} = m_{ex} / V_{ex}$ (mol.m^{-3}), and transfers into the cell interior to a concentration $C_{in} = m_{in}/ V_{in}$ dominantly due to diffusion according to Fick's law, if there is no ionic charge on the drug considered.[57]

$$J = dm_{in} /A_p{\cdot}dt = (D/d_M) \cdot(C_{ex} - C_{in}) \qquad \text{Eq. 2.29}$$

where

J is the flux of the drug through the cell membrane (mole.m^{-2}.s^{-1});
D is the diffusion coefficient of the drug (m^2/s);
d_M is the thickness of the cell membrane;
A_p is the total electro-pore area = $K \cdot \pi \cdot r_p^2$.

Eq. 2.30 thus gives the flow rate of the drug that enters a cell by diffusing through the aqueous pores across the cell membrane

$$dm_{in} /dt = (D/d_M) \cdot(C_{ex} - C_{in})\cdot K \cdot \pi \cdot r_p^2 \cdot e^{-t/\tau_p} \qquad \text{Eq.2.30}$$

where

K is the total number of pores;
r_p is the average pore radius.

$C_{ex} >> C_{in}$ and C_{ex} are assumed to be approximately constant during the period T of drug accumulation to the cell (mole per cell) according to Eq. 2.31,

$$m_{in} = (D/d_M) \cdot C_{ex} \cdot K \cdot \pi \cdot r_p^2 \cdot \tau_p \cdot (1 - e^{-T/\tau_p}) \qquad \text{Eq.2.31}$$

According to diagram D of *Figure 8* in the work of Krassowska and Filev (2007), the total area of pores ($K \cdot \pi \cdot r_p^2$) as a fraction of the cell surface

(A_{cell}) is approximately rather constant, with a value of about $7 \cdot 10^{-4}$ at field strength above 400 V/cm.[55] This is in agreement with the results of an experimental study on the outcome of electro-enhanced chemotherapy, which suggested that the uptake ratio depends mostly on the pulse length and number of applied pulses.[58]

$$total\ pore\ area: K \cdot \pi \cdot r_p^2 = A_{cell} \cdot 7 \cdot 10^{-4}$$

$$m_{in} = (D/d_M) \cdot C_{ex} \cdot A_{cell} \cdot \tau_p \cdot 7 \cdot 10^{-4} \cdot (1 - e^{-T/\tau_p}) \qquad \text{Eq.2.32}$$

Figure 2-13 displays the diffusion coefficients set against the molecular weight for compounds in the range of 135 and 68000 fitted to Eq. 2.33.[59,60]

$$D = 1.05 \cdot 10^{-8}[Mw.]^{-0.484} \qquad \text{Eq. 2.33}$$

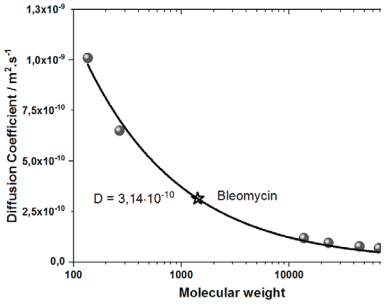

Figure 2-13
Variation of the diffusion coefficients with the molecular weight

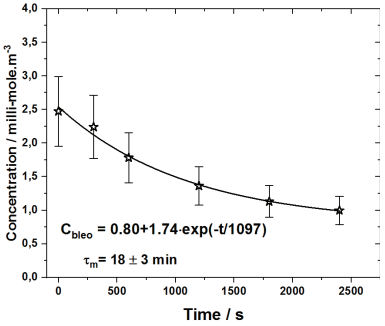

Figure 2-14
Average of plasma concentration of Bleomycin after intravenous bolus injection of a mean dose of 26000 IU Bleomycin in 24 patients older than 65.[61]

With a molecular weight of 1415.5 for Bleomycin in Eq. 2.33, we see a value of $D = 3.14 \cdot 10^{-10}$ m^2.s^{-1} for this drug. The average radius for a tumour cell is considered to be about 50 µm.[55] Thus, the area ($A=4\pi r^2$) of the tumour cell is about $3.14 \cdot 10^{-8}$ m^2. The thickness of the cell membrane d_M is considered to be 7 nm.[62]

Intravenous administration of Bleomycin is recommended by ESOPE as a dose of 15000 IU per m^2 body surface, 15 000 IU (International Units) = 15 USP units = 1mg. Administration of 15 mg of Bleomycin thus corresponds to about 10 micromole ($1.06 \cdot 10^{-5}$ mole). Eq. 2.34 for the excretion of Bleomycin is estimated using data from a clinical study of intravenous bolus injections of a mean dose of 26000 IU of Bleomycin.[61]

Figure 2-14 shows the average of Bleomycin plasma concentration after an intravenous bolus injection of a mean dose of 26000 IU of Bleomycin in 24 patients older than 65 a.[58] The elimination curve of Bleomycin plasma concentration values converted to milli-mole per m^3 presented in Figure 2-14 fitted to an exponential decay gives us the following equation:

$$C_{Bleo} = 0.80 + 1.74 \cdot e^{-t/1097}\ [\text{millimole}.m^{-3}\ ] \qquad \text{Eq.2.34}$$

The mean elimination time is 1097 s, which corresponds to τ_m= 18 ± 3 min and the halftime $\tau_{½}$=12.5±2 min. About 8 minutes after the bolus injection of Bleomycin, the electro pulse treatment is performed. At this time, the Bleomycin plasma concentration is about $2{\cdot}10^{-3}$ mole.m^{-3} and, after about T = 20 s, the total uptake of Bleomycin in the cell became $1{\cdot}10^{-14}$ mole per cell according to Eq.2.35. Considering that the Avogadro's Constant is $6.022{\cdot}10^{23}$ molecules.$mole^{-1}$, the Bleomycin uptake is equivalent to $6{\cdot}10^{9}$ molecules per cell.

$$m_{in} = (3.14{\cdot}10^{-10}/7{\cdot}10^{-9}) \cdot 2{\cdot}10^{-3}{\cdot}(3.14 \cdot 10^{-8}) \cdot 5 \cdot (7 \cdot 10^{-4}) \qquad \text{Eq. 2.35}$$

$$m_{in} = 1 \cdot 10^{-14}\ \text{(mole per cell)}$$

CHAPTER III

ELECTRO-DOSIMETRY

3.1 INTRODUCTION

External electric fields applied to biological materials interact directly wiy free electric charges such as electrons and ions, as well as on ionic groups in larger molecules. They also interact with dipoles such as water, and induce dipoles in molecules with polarizable groups. The cell membranes seem to be the critical target for interaction, causing intra-molecular transitions and intermolecular processes that lead to structural reorganization of the cell membranes.

Applying high voltage pulses to cells in cultures or in tissue causes various degrees of structural reorganisations in the cell membranes, which might end with a dielectric collapse or breakdown. This condition is either called "*electroporation"* or "*electro-permeabilization*", and can be either reversible or irreversible depending on the characteristics of the applied voltage pulses.[5]

In such a transient state, the cell membrane becomes permeable to compounds that generally do not pass this barrier into the cytoplasm of the cell. Direct transfer of DNA plasmids, and other nucleic acids (such as RNA), proteins, and other molecules into cells and microorganisms use that condition. Another possibility is that neighbouring cells with membranes in a transient state might fuse their membranes and form a new giant cell. These properties of membranes in a transient state have led to electrical field pulse techniques that are gaining increasing importance in cellular and molecular biology, in gene technology, and in various medical therapeutic procedures.[24,63]

3.2 MODELLING OF THE IMPEDANCE OF TISSUE

3.2.1 MODEL OF THE CELL MEMBRANE

Figure 3-1 shows a simple model of the cell membrane, where R_m is the resistivity and C_m is the capacitance of the cell membrane.

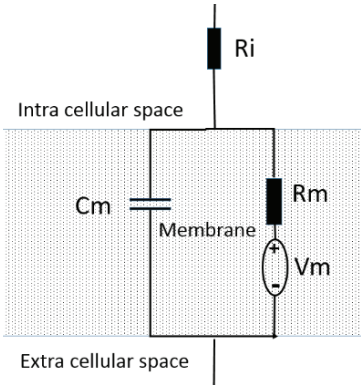

Figure 3-1
An equivalent circuit of a cell membrane Rm is the resistivity and Cm is the capacitance of the cell membrane.

Vm is the voltage created by the ions Na^+ K^+ and Cl^- covering the surface of the membrane.

An externally applied voltage dV during the time dt over the membrane "*m"* induces a charge $dQ = C_m \cdot dV$ in the capacitor that creates the current

$$i_m = dQ/dt = C_m \cdot dV/dt = (V\text{-}V_m)/R \qquad \text{Eq.3.1}$$

Thus, $dV/dt = (V\text{-}V_m)/R_mC_m$, which is a first order kinetic equation with the rate constant $1/R_mC_m$ or the time constant $\tau_m = R_mC_m$. The impedance of the cell membrane Z_m is derived by the parallel resistance $Z_r = R_m$ and capacitance $Z_c = -j/\omega \cdot C_m$, where $j = \sqrt{-1}$ is the imaginary unit, $\omega = 2\pi \cdot f$, and f is the frequency in Hz.

$$\frac{1}{Z_m} = \frac{1}{R_m} + \frac{1}{Z_c} = \frac{1}{R_m} + j \cdot \omega \cdot C_m = G_m + j \cdot \omega \cdot C_m = Y; \qquad \text{Eq.3.2}$$

where G is the conductance and Y is called the admittance:

$$Z_m = \frac{R_m}{1 + j \cdot \omega \cdot C_m \cdot R_m} = \frac{R_m \cdot (1 - j \cdot \omega \cdot C_m \cdot R_m)}{(1 + j \cdot \omega \cdot C_m \cdot R_m) \cdot (1 - j \cdot \omega \cdot C_m \cdot R_m)} = \frac{R_m}{1 + \omega^2 \cdot C_m^2 \cdot R_m^2} - \frac{j \cdot \omega \cdot C_m \cdot R_m^2}{1 + \omega^2 \cdot C_m^2 \cdot R_m^2} \qquad \text{Eq.3.3}$$

$$Z_m = Re[Z_m] + j \cdot Im[Z_m] \qquad \text{Eq.3.4}$$

The resistance is the real part of the membrane impedance:

$$Re[Z_m] = \frac{R_m}{1 + \omega^2 \cdot C_m^2 \cdot R_m^2} \qquad \text{Eq.3.5}$$

The reactance is the imaginary part of the membrane impedance:

$$Im[Z_m] = -\frac{\omega \cdot C_m \cdot R_m^2}{1 + \omega^2 \cdot C_m^2 \cdot R_m^2} \qquad \text{Eq.3.6}$$

The modulus or absolute value of the membrane impedance is $|Z_m| = \sqrt{Z_m \cdot Z_m^*}$, where Z_m^* is the complex conjugate (j replaced with –j) of the membrane impedance.

$$Z_m = Re[Z_m] + j \cdot Im \; ;$$

$$|Z_m| = \sqrt{Re[Z_m]^2 + Im[Z_m]^2} \; ; \qquad \text{Eq.3.7}$$

$$|Z_m| = \frac{R_m \cdot \sqrt{1+\omega^2 \cdot C_m^2 \cdot R_m^2}}{1+\omega^2 \cdot C_m^2 \cdot R_m^2} \; ;$$

The characteristic time constant of the membrane is $\tau_m = R_m \cdot C_m$, and thus the characteristic frequency $f_c = 1/\tau_m = 1/ R_m \cdot C_m$. The time-constant τ_m is in the order of 1-10ms for a typical biological membrane, and thus the characteristic frequency is in the order of 0.1-1 kHz.

The equation for the impedance Z_m in terms of the time-constant τ_m:

$$Z_m = \frac{R_m}{1+j \cdot \omega \cdot \tau_m} = \frac{R_m}{1+\omega^2 \cdot \tau_m^2} - \frac{j \cdot \omega \cdot \tau_m \cdot R_m}{1+\omega^2 \cdot \tau_m^2} \quad \text{and}$$

$$|Z_m| = \frac{R_m \cdot \sqrt{1+\omega^2 \cdot \tau_m^2}}{1+\omega^2 \cdot \tau_m^2} \qquad \text{Eq.3.8}$$

At low frequency $f \approx 0$. $Z_m = R_m$, and at very high-frequency $f \to \infty$ $Z_m \to 0$, the admittance Y is defined in Eq. 3.9:

$$Y = G + j \cdot \omega \cdot C \quad \text{where} \quad G = \frac{A}{d} \cdot \sigma \text{ and } C = \frac{A}{d} \cdot \varepsilon \qquad \text{Eq.3.9}$$

Where G is the conductance (Ω^{-1}); σ (S) is the conductivity; C (F) is the capacitance; and ε is the permittivity of a membrane with thickness d (m) between two plate electrodes of cross-section A (m^2). The capacitance of a patch of a typical biological membrane is about 1 μF/cm^2 (10 mF/m^2).

3.2.2 MODEL OF THE CELL

Figure 3-2 displays the frequency dependence of cell resistance ($Re[Z_m]$) derived from the equations for the impedance of the cell $Z_c = R_i + Z_m$, where Z_m is the impedance of the membrane. These equations for the calculation of the Nyquist Plot are shown in Figure 3-3.

$$Z_m = \frac{R_m}{1+j \cdot \omega \cdot \tau_m} = \frac{R_m}{1+\omega^2 \cdot \tau_m^2} - \frac{j \cdot \omega \cdot \tau_m \cdot R_m}{1+\omega^2 \cdot \tau_m^2} \qquad \text{Eq.3.10}$$

$$Z_c = R_i + \frac{R_m}{1+\omega^2 \cdot \tau_m^2} - \frac{j \cdot \omega \cdot \tau_m \cdot R_m}{1+\omega^2 \cdot \tau_m^2} = \frac{R_i + R_m + \omega^2 \cdot \tau_m^2 \cdot R_i}{1+\omega^2 \cdot \tau_m^2} - \frac{j \cdot \omega \cdot \tau_m \cdot R_m}{1+\omega^2 \cdot \tau_m^2} \qquad \text{Eq.3.11}$$

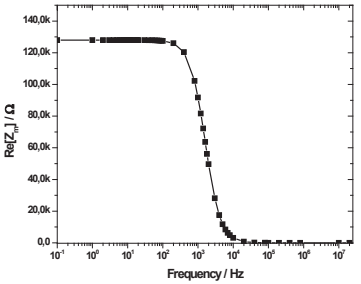

Figure 3-2
Frequency dependence of cell resistance = *Re*[Z_m], C_m=7.81·10^{-10} F; τ_m = 10^{-4} s; R_m = 128kΩ; Ri = 300 Ω

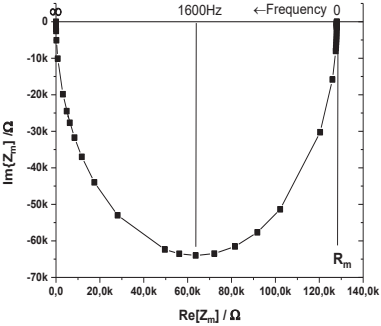

Figure 3-3
Nyquist Plot of the reactance = *Im*[Z_m] versus resistance of the cell with Ri = 300 Ω; C_m=7.81·10^{-10} F; τ_m = 10^{-4} s; Rm = 128kΩ

At low frequency, $f \approx 0$ $Z_c = R_m + R_i$, and, at very high-frequency, $f \rightarrow \infty$ $Z_c \rightarrow R_i$.

3.2.3 IMPEDANCE MODEL OF TISSUE

Figure 3-4 shows a simple impedance model of intact tissue-assuming cells surrounded by a cell membrane of infinite high resistance and a capacitance of C_m. The interstitial fluid of the cells has a resistance of R_i. An extracellular fluid of resistance R_x fills the space between separate cells in the tissue.

Exposure of tissue to pulsed electric fields changes the conductivity of the cell membrane. This process is often called electroporation because one believes that the membrane is perforated and, through the ions, moves freely through the pores. The intact cell membrane has a very high resistance that decreases considerably after exposure to the pulsed electric field.

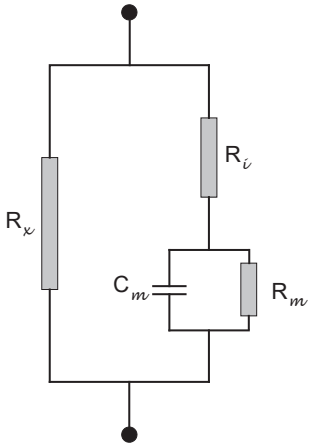

Figure 3-4
The equivalent circuit of tissue where R_x is the resistivity of the extracellular fluid, R_i is the resistivity of intracellular fluid, R_m is the resistivity of the cell membrane, and C_m is the capacitance of the cell membrane. The impedance of the cell is $Z_c = R_i + Z_m$

$$\frac{1}{Z_m} = \frac{1}{R_m} + j \cdot \omega \cdot C_m \quad \omega = 2\pi \cdot f \qquad \text{Eq.3.12}$$

$$|Z_m| = \frac{1}{\sqrt{\left(\frac{1}{R_m}\right)^2 + (\omega \cdot C_m)^2}} = \frac{R_m}{\sqrt{1 + (\omega \cdot R_m C_m)^2}} \qquad \text{Eq.3.13}$$

$$|Z_m| = \frac{R_m}{\sqrt{1 + (\omega \cdot \tau_m)^2}} \qquad \text{Eq.3.14}$$

The impedance of the whole tissue derives from the expression for adding parallel circuits of impedance:

$$\frac{1}{Z_{tis}} = \frac{1}{R_x} + \frac{1}{Z_c} = \frac{1}{R_x} + \frac{1}{R_i + Z_m} = \frac{R_i + Z_m + R_x}{R_x \cdot (R_i + Z_m)} \qquad \text{Eq.3.15}$$

$$Z_{tis} = \frac{R_x \cdot (R_i + Z_m)}{R_i + Z_m + R_x} = \frac{R_x \cdot Z_c}{Z_c + R_x} = \frac{R_x \cdot (Z_c^{RE} + j \cdot Z_c^{IM})}{(Z_c^{RE} + j \cdot Z_c^{IM}) + R_x} = \frac{R_x \cdot (Z_c^{RE} + j \cdot Z_c^{IM})}{(Z_c^{RE} + R_x) + j \cdot Z_c^{IM}} \qquad \text{Eq.3.16a}$$

$$Z_{tis} = \frac{R_x \cdot (Z_c^{RE} + j \cdot Z_c^{IM}) \cdot [(Z_c^{RE} + R_x) - j \cdot Z_c^{IM}]}{[(Z_c^{RE} + R_x) + j Z_c^{IM}] \cdot [(Z_c^{RE} + R_x) - j \cdot Z_c^{IM}]} = \frac{R_x \cdot \left[(Z_c^{RE} + R_x) \cdot Z_c^{RE} + (Z_c^{IM})^2 - j \cdot Z_c^{IM} Z_c^{RE} + (Z_c^{RE} + R_x) j \cdot Z_c^{IM}\right]}{\left[(Z_c^{RE} + R_x)^2 + (Z_c^{IM})^2\right]} = \qquad \text{Eq.3.16b}$$

$$Z_{tis} = \frac{R_x \cdot [(Z_c^{RE} + R_x) \cdot Z_c^{RE} + (Z_c^{IM})^2]}{[(Z_c^{RE} + R_x)^2 + (Z_c^{IM})^2]} \qquad \text{Eq.3.16c}$$

$$+j\cdot\frac{R_x\cdot[(Z_c^{RE}+R_x)\cdot Z_c^{IM}-Z_c^{IM}\cdot Z_c^{RE}]}{[(Z_c^{RE}+R_x)^2+(Z_c^{IM})^2]}$$

At low frequency, $f \approx 0$ $Z_c^{RE} = R_m + R_i$, $Z_c^{IM} = 0$, and since $R_m >> R_i$ and R_x:

$$Z_c^{RE}(0) = \frac{R_x\cdot(R_m+R_i)}{R_m+R_i+R_x} = R_x \qquad \text{Eq.3.17}$$

and at very high-frequency, $f \to \infty$ $Z_c^{RE} \to R_i$ $Z_c^{IM} \to 0$, thus:

$$Z_c^{RE}(\infty) = \frac{R_x\cdot R_i}{R_i+R_x} \qquad \text{Eq. 3.18}$$

The following equation (3.19) can thus estimate the resistivity of the intracellular fluid R_i in intact tissue:

$$R_i = \frac{Z_c^{RE}(\infty)\cdot R_x}{R_x - Z_c^{RE}(\infty)} \qquad \text{Eq. 3.19}$$

The effect of the application of pulsed electric fields is assumed to permeabilize the cell membrane which decreases the impedance mainly at a frequency region below 10 kHz.

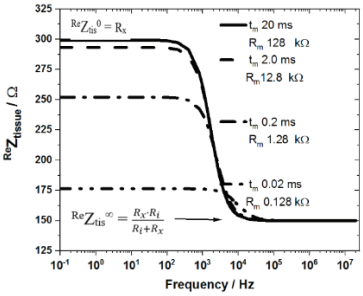

Figure 3-5
Frequency dependence of tissue resistance = Re[Z_{tissue}]; τ_m = 0.02-20ms; R_m = 128 - 0.128 kΩ; R_i = R_x = 300 Ω.

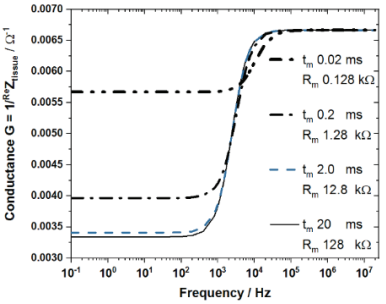

Figure 3-6
Frequency dependence of tissue conductance G = 1/Re[Z_{tissue}]; τ_m = 0.02- 20ms; R_m = 128 - 0.128 kΩ; R_i = R_x = 300 Ω.

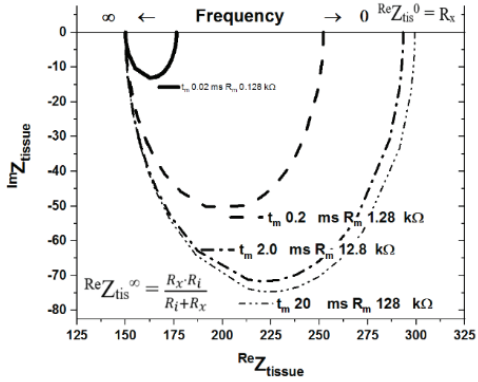

Figure 3-7
Nyquist Plot of the reactance = *Im*[Z_m] versus resistance of tissue model with τ_m = 0.20-20 ms; R_m = 128 - 0.128 kΩ; R_i = R_x = 300 Ω.

Complex parameters express the admittance Y with a capacitor, the dielectric constant of which (ε) represents its ability to store electrical energy.

$$Y^* = j \cdot \omega \cdot C^* = j \cdot \omega \frac{A}{d} \cdot \varepsilon^* = j \cdot \omega \frac{A}{d} (\varepsilon - j \cdot \varepsilon) = \quad \text{Eq.3.20}$$

$$= j \cdot \frac{A}{d} (\varepsilon \cdot \omega - j \cdot \omega \cdot \varepsilon) = G + j \cdot \omega \cdot C$$

Thus, the conductance is expressed as:

$$G = \frac{A}{d} \cdot \sigma \equiv \frac{A}{d} \cdot \omega \cdot \varepsilon'' \text{ and therefore } \varepsilon'' = \frac{\sigma}{\omega}.$$

and the relative permittivity: $$\varepsilon_r'' = \frac{\varepsilon''}{\varepsilon_0} = \frac{\sigma}{\varepsilon_0 \cdot \omega};$$

The loss angle δ of a capacitor is defined so that the ideal capacitor with zero losses has a zero loss angle, which means that δ = 90° - θ, where θ is the recorded phase angle. Thus, $\tan\delta = 1/\tan\theta$. The *loss tangent, tan* δ, is also called the *dissipation factor*, and is equivalent to the energy loss per cycle divided by the energy stored per cycle (RMS or peak value).

The impedance of the equivalent circuit is $Z = |Z| \cdot \cos\theta + j \cdot |Z| \cdot \sin\theta$, and $\tan\theta = \frac{Im[Z]}{Re[Z]}$.

The following equation gives the loss tangent for a cell membrane

$$\tan\delta = 1/\tan\theta = \frac{Re[Z]}{Im[Z]}. = -1/\omega \cdot C_m \cdot R_m = 1/\omega \cdot \tau_m \quad \text{Eq.3.21}$$

The power loss in the circuit only takes place in the resistive part Re[Z] if the capacitive part is an ideal capacitor. The frequency characteristic depends on the power loss of the circuit. With the constant amplitude

voltage U, the power loss moves from zero levels at very low frequencies to a defined value U^2/R at high frequencies. With a constant amplitude current, the power level moves from a constant value at very low frequencies through a maximum at the frequency determined by the time constant $\tau = RC$ and to zero at high frequency.

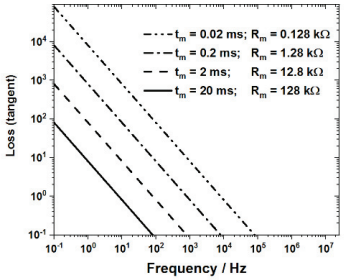

Figure 3-8
Loss tangent for the cell membrane τ_m = 0.02-2ms; R_m = 128 - 0.128 kΩ.

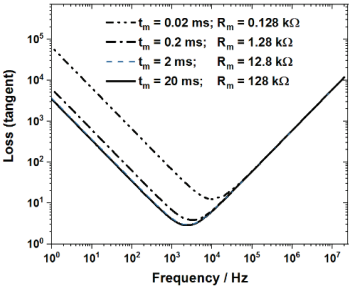

Figure 3-9
Loss tangent tan δ as a function of frequency of the tissue model with R_m = 128 - 0.128kΩ; R_i=R_x= 300Ω;

In Figure 3-8, the values of $\tan\theta = \frac{Im[Z]}{Re[Z]}$ multiplied by 1000 of the tissue model of Figure 3-9, with $\tau_m = R_m \cdot C_m$ varied from 0.02-20ms and R_m, varied from 128 to 0.128kΩ to simulate the effect of electroporation. Figure 3-9 shows that the minimum value of tanθ decreases and shifts towards higher frequencies by decreasing the value of R_m. The ratio of the loss-tangent at a specific frequency before and after exposure of the tissue to pulsed electric fields applies as a dosimetry quantity. This parameter is called the "loss factor" and is evaluated as shown in Eq.3.22. It depends on the power of the exposure that controls the process of electroporation.

$$Loss\ Factor = \left(\frac{\tan\theta_{after}}{\tan\theta_{before}}\right) = \left|\frac{\tan\delta_{before}}{\tan\delta_{bafter}}\right| \qquad \text{Eq.3.22}$$

The loss factor measured at about 1 kHz to record the effect of exposure to a pulsed electric field is 1 if there is no change in the phase angle after pulse exposure. In case of extreme powerful exposure, the value of the recorded loss factor of the exposed object tends to approach zero. Therefore, this results in another quantity, the "loss change index" (LCI), which is defined as 1- loss factor and shown in the following Eq. 3.23.

$$LCI = \left(1 - \frac{\tan\theta_{after}}{\tan\theta_{before}}\right) \qquad \text{Eq.3.23}$$

By this definition, the loss change index is 0 if there is no change in the phase angle after pulse exposure, and is equal to 1 when the phase angle after exposure approaches 0, meaning that the loss tangent becomes infinite.

3.3 EVALUATION OF MODEL PARAMETERS USING IMPEDANCE DATA

Direct electrical currents (DC) and low-frequency currents are limited to the extracellular space. With the increasing frequency of the alternating current, the impedance of the cell membrane decreases, and the current travels straight through the cells, as demonstrated in Fig. 3-10. Electrical impedance readings over a frequency range then allow the separation of extracellular space and the membrane.

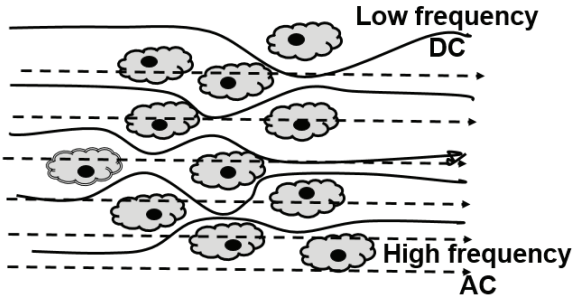

Figure 3-10
Electrical DC and low-frequency current travel in the extracellular space between the cells (represented by the solid line), while high-frequency AC-current (represented by the dashed line) passes the cell membrane and travels straight through the cells.

Electro-permeabilization is a membrane-associated process which affects the membrane resistance to direct electrical currents. From experiences of electro-permeabilization, the situation in vivo appears to be more complex than shown by the model of the single cell membrane, which resembles an ultra-thin capacitor of high resistance that envelops the intracellular fluids, as shown in Figure 3-4 in the modelling section.

3.3.1 IMPEDANCE MEASUREMENTS

In Figure 3-11, we can see the results from an experiment with impedance measurements in the thigh of a rat both before and 4 minutes after electro-pulse treatment with 8 exponential pulses of 1200 V/cm with a time-constant of 1ms. To simulate the drug administration, a volume of 0.2ml of saline was administered intramuscularly into the target area.

Figure 3-12 shows the impedance difference of measurements in the frequency interval 15 - 200 000 Hz in the thigh of a rat before and 4 minutes after electro-pulse treatment with eight exponential pulses of various field strengths (V/cm) with a time constant of 1ms.

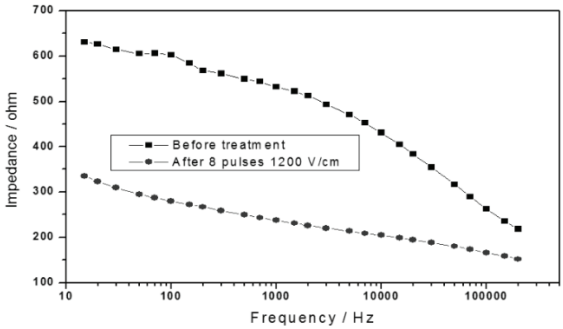

Figure 3-11
Results from an experiment with impedance measurements in the thigh of a rat both before and 4 minutes after electroporation treatment with 8 exponential pulses of initial amplitude of 1200V/cm with a time-constant of 1ms. Saline (0.2ml) was injected intramuscularly 4 minutes before the first measurement.

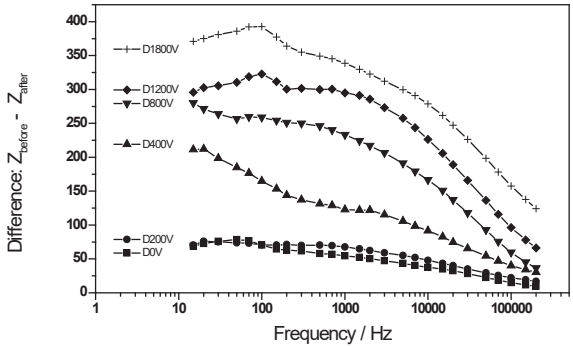

Figure 3-12
The difference of impedance measurements in the frequency interval 15Hz – 200kHz in the thigh of a rat before and 4 minutes after electro-pulse treatment with 8 exponential pulses of various initial amplitude D(V) with a time constant of 1ms. Saline (0.2 ml) was injected intramuscular 4 minutes before the first measurement.

Figure 3-13a displays the conductance G (Ω^{-1}) at various frequencies before (0) and after applying 2, 4, 8, 12, and 20 exponential pulses of initial field strength 1 200 V/cm, and τ =1.0ms to the muscles of rats. The corresponding conductance ratio G_{after}/G_{before} is displayed in Figure 3-13b.

As seen in Figure 3-13b, the conductance ratio G_{after}/G_{before} shows a maximum in the frequency range 1 - 50 kHz. At higher frequencies, however, the membrane resistance is short-circuited, and the electric field lines pass more uniformly through the tissue structure, and less change in the conductivity caused by the applied pulses occurs.

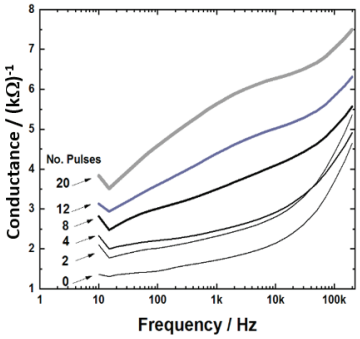

Figure 3-13a
Conductance G after 0, 2, 4, 8, 12, and 20 exponential pulses of initial field strength 1200 V/cm, and τ =1.0ms.

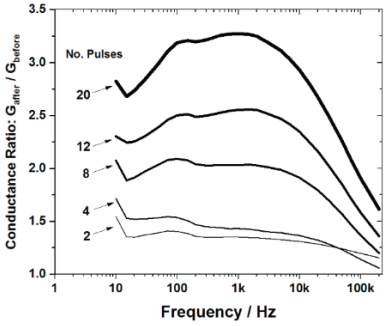

Figure 3-13b
Conductance ratio, G_{after} / G_{before} for 2, 4, 8, 12 and 20 exponential pulses of initial field strength 1200 V/cm, τ =1.0ms.

At low frequency $f \approx 0$, the following equation expresses the low frequency impedance $Z_{tis}^{RE}(LF)$ of the tissue:

$$Z_c^{RE} = \mathrm{R_m} + \mathrm{R_i}\ ,\ Z_c^{IM} = 0 \quad \text{thus} \lim_{f\to 0} Z_{tis}^{RE} = \frac{R_x \cdot (R_m + R_i)}{R_m + R_i + R_x} = \mathrm{R_x} = Z_{tis}^{RE}(LF) \qquad \text{Eq.3.24}$$

At high frequency $f \to \infty$, the following equation expresses the high frequency impedance $Z_{tis}^{RE}(HF)$ of the tissue:

$$Z_c^{RE} \to \mathrm{R_i}\ Z_c^{IM} \to 0 \text{ thus } \lim_{f\to\infty} Z_{tis}^{RE} = \frac{R_x \cdot R_i}{R_i + R_x} = Z_{tis}^{RE}(HF) \qquad \text{Eq.3.25}$$

The resistivity of the intracellular fluid R_i in intact tissue can thus be estimated by the equation:

$$R_i = \frac{Z_{tis}^{RE}(HF)\ \cdot R_x}{R_x - Z_{tis}^{RE}(HF)} \qquad \text{Eq.3.26}$$

By assuming $\mathrm{R_i} \approx \mathrm{R_x} = \mathrm{R}$; $Z_{tis}^{RE}(HF) = R/2$; and

$$Z_{tis}^{RE}(LF) = \frac{R \cdot (R_m + R)}{R_m + 2R} \approx \mathrm{R}; \qquad \text{Eq.3.27}$$

3.3.2 RESISTIVITY CHANGE INDEX, RCI

Equation 3.28 defines the resistivity change index (RCI) as the relative decrease in resistivity after electroporation or the relative increase in conductance G =1/R, RCI=0, if there is no change (i.e. R_{after}=R_{before}).

$$RCI = \left(1 - \frac{R_{after}}{R_{before}}\right) = \left(1 - \frac{G_{before}}{G_{after}}\right) \qquad \text{Eq.3.28}$$

where

R_{before} is the resistivity (Ω) measured before the first pulse.
R_{after} is the resistivity after electroporation with N_p pulses.

If no change occurs, RCI = 0. If substantial changes occur, RCI → 1.

3.3.3 LOSS TANGENT AND LOSS CHANGE INDEX

Figures 3-14a and 3-14b show the loss tangent recorded in rat muscle tissue before and after applying 0, 1, 4, and 8 rectangular pulses of 1000 V amplitude and 0.1ms pulse-length, and Figures 3-15a and 3-15b display the *loss change index* calculated according to Eq. 3.23.

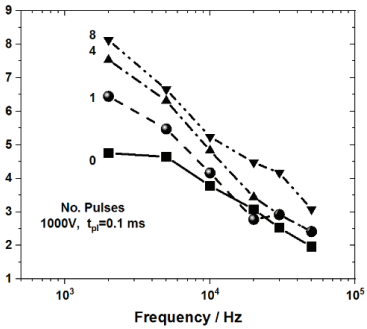

Figure 3-14a
Loss tangent after 0, 1, 4, and 8 rectangular pulses of E=1000 V/cm, PL =0.1ms,

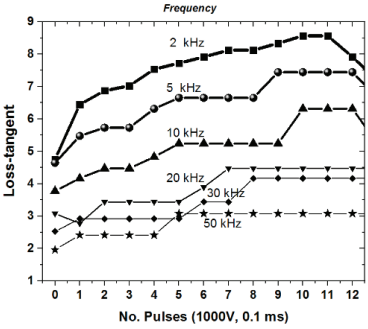

Figure 3-14b
Loss tangent recorded at various frequencies for various numbers of rectangular pulses of E=1000 V/cm, PL =0.1ms.

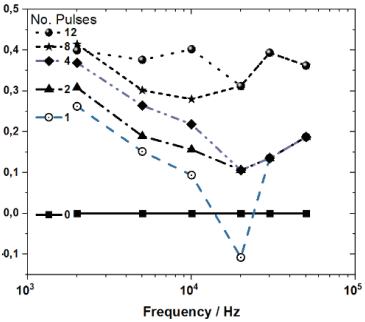

Figure 3-15a
Loss change index after 0, 1, 3, 4, 8, and 12 rectangular pulses of E=1000 V/cm, PL =0.1ms.

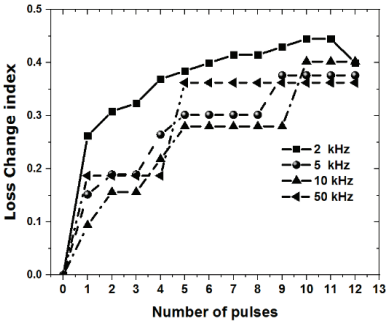

Figure 3-15b
Loss change index recorded at various frequencies for various numbers of rectangular pulses of E=1000 V/cm, PL =0.1ms.

3.3.4 CONDUCTANCE RELAXATION

Figure 3-16 shows the ratio of conductance after each pulse against the conductance before pulse treatment. The conductance ratio $R_G^N = G_N/G_0$ steadily increases after each applied pulse of 1000 V/cm and 0.1ms pulse length. After the last pulse, N=12, the conductance ratio $R_G^{12} = R_G^{end}$ begins to decrease due to the recovery of the permeabilized membrane. However, only a fraction of the conductance ratio R_G^{end} is reversible, and after some

time the conductance ratio reaches a plateau value indicating the conductance ratio of irreversible permeabilization R_G^{end}(irr).

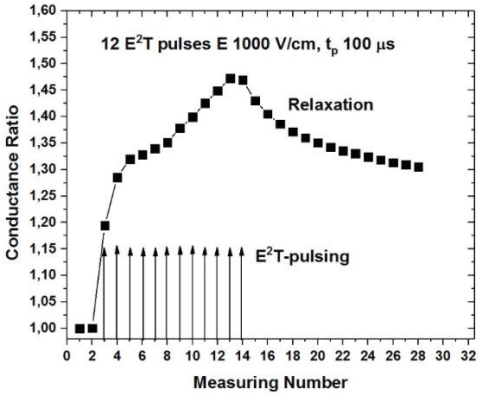

Figure 3-16
Conductance ratio G_{afterN}/G_0 recorded at 2 kHz before, during and after 12 consecutive EP pulses of 1000 V/cm amplitude and 100 µs pulse length.

The relaxation of the conductance ratio at the end of electro-pulsing R_G^{end} follows a first order differential equation:

$$\frac{dR_G^{end}}{dt} = -k_{relax} \cdot R_G^{end} \qquad \text{Eq.3.29}$$

Where

k_{relax}	is the rate constant of the conductance relaxation
R_G^{end}	is the conductance ratio at the end of electro-pulsing
$R_G^{\infty}(irr)$	is the conductance left after its relaxation

$$R_G^{end}(t) = R_G^{\infty}(irr) + \{R_G^{end}(0) - R_G^{\infty}(irr)\} \cdot e^{-k_{relax} \cdot t} \qquad \text{Eq.3.30}$$

- The fraction of reversible electro permeabilization f_{rev} is equal to $\{R_G^{end}(0) - R_G^{\infty}(irr)\}/R_G^{end}(0)$, and;
- The fraction of irreversible electro-permeabilization $f_{irr} = (1- f_{rev})$.

The data displayed in Figure 3-16 yield the following values for f_{rev} and f_{irr}:

- $f_{rev} = (1.455 – 1.30) / 1.455 = 0.107$, and;
- $f_{irr} = 0.893$.

3.3.5 CONDUCTANCE RELAXATION AFTER EACH PULSE

Experiments performed with 12 DC-pulses of 100 V amplitude and 20ms pulse length delivered with 167 ms intervals were conducted through electrodes of 1mm diameter, and inserted in the thigh rat muscle at a distance of 8mm. The upper diagram in Figure 3-17 presents the ratio $R_G^N = G_N/\overline{G_0}$ of the recorded conductance values G_N relative to the average value $\overline{G_0}$, before pulsing.

The second diagram in Figure 3-17, on the other hand, shows the consecutive difference $R_G^N - R_G^{N-1}$ of the progressive conductance values. The conductance ratio increases most rapidly after the first pulse, before gradually increasing more slowly.

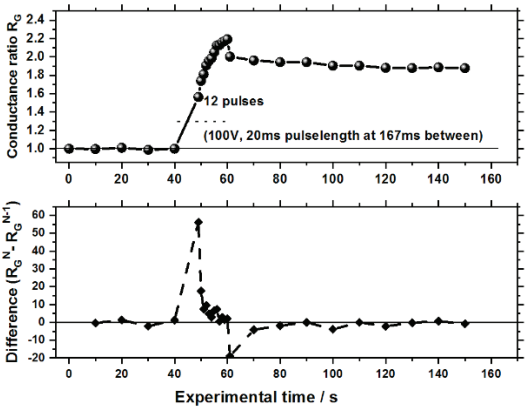

Figure 3-17
The first diagram above shows the conductance value relative to the average before pulsing, after each of the 12 pulses with 100 V amplitude and 20ms pulse-length, and with 10 measurements conducted after the last pulse. The second diagram shows the consecutive difference of the conductance R_G.

Figure 3-18 shows the results of another experiment exposing rats to 6 DC pulses of 100 V amplitude and 20ms pulse length delivered with 167ms intervals with four measurements of conductance performed before the first EP pulse and at 50ms after each next EP pulse.

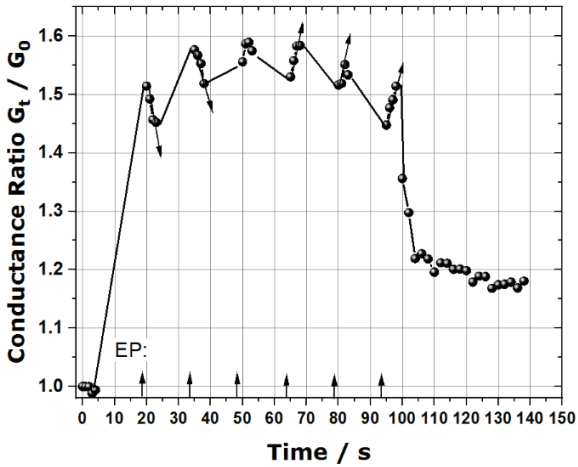

Figure 3-18
The conductance relative to the first measured value before any treatment with pulses of 100 V amplitude and 20ms pulse-length. The arrows at the bottom indicate the execution of the electro-pulses (EP). Four conductance measurements of 28ms are performed before the first EP pulse and at 50ms after each next EP pulse.

The first measuring pulses indicate a slight conductance increase probably due to primary electrode polarization processes. The first electro-pulse (EP) causes a 35% increase in conductance, and the following conductance measurements indicate a relaxation of the opened pores that cause a decrease of the conductance. The next EP pulse just compensates for the relaxation. The 4th and following pulses, however, cause an increasing conductivity, probably due to tissue destruction causing swelling and disruption of cells. After the last EP pulse, the conductance relaxes in two exponential steps to a level of about 1.18, that is the level due to non-reversible electroporation.

3.4 ELECTRICAL CONDUCTIVITY OF TISSUE

HEALTHY TISSUE

The data on the electrical conductivity of human tissues used in electro-dosimetry are drawn from both in vitro and ex vivo measurements. In vivo tissues may differ from in vitro and ex vivo states due to the various degrees of blood perfusion in living organs.[64,65,66] Recent studies with magnetic resonance imaging (MRI) combined with electrical impedance tomography

provide information about the in vivo conductivity of human tissues at DC or below the 1 kHz frequency range.[64]

Andreuccetti and Fossi (2000) developed a client-server approach for determining the dielectric properties of human body tissues in the frequency range from 10 Hz to 100 GHz using the parametric model and the parameter values previously developed by Gabriel and colleagues (1996).[67,66,68,69]

The webpage http://www.dtic.mil/dtic/tr/fulltext/u2/a305826.pdf recalls the definitions and underlying theory of the dielectric properties of biological tissues. Mathematical and computational codes and various online and offline applications are presented, based on Gabriel's parametric model.[67,66,68,69]

The server program (found on http://niremf.ifac.cnr.it/tissprop/htmlclie/htmlclie.php) runs in the background on a central system and is responsible for the management of the parameter database (14 parameters for each defined tissue) and the calculation of the dielectric properties of requested tissues at requested frequencies. These properties comprise relative permittivity, electrical conductivity, and a few significant derived quantities, such as the loss tangent, the wavelength, the penetration, and skin depth.

The user executes the client program using a remote system, connected to the server by a local or a wide area network supporting the TCP/IP protocol. The client program implements the user interface and translates user requests into commands for the server. The program also deals with the reception and display of the requested data.

The main advantage of the client/server approach consists in the centralized management of the tissue parameters, allowing new tissues to be defined and changing parameter values, without having to redistribute the software to the users. The users, on their side, also get rid of the need to implement the code for the calculation of the dielectric properties from the parameter values.[1]

Figure 3-19 shows relative permittivity in the frequency region 1 Hz – 1 GHz for muscle tissue derived from the ITIS database. Figures 3-20 shows the conductivity in Siemens per m ($S.m^{-1}$) in the frequency region 1 Hz – 1 GHz for muscle tissue derived from the ITIS database.

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[1] (Internet document;
URL: http://www.dtic.mil/dtic/tr/fulltext/u2/a305826.pdf (authorized mirror at http://niremf.ifac.cnr.it/docs/dielectric/home.html). See Appendix C, for the modelling of the data (authorized mirror here).[70,71,65,68,69,67,72]
Documents available online at the address:
http://niremf.ifac.cnr.it/tissprop/document/tissprop.pdf
The Foundation for Research on Information Technologies in Society (ITIS) database for the dielectric properties of various human tissues can be reached at the web address: https://www.itis.ethz.ch/virtual-population/tissue-properties/database/dielectric-properties/

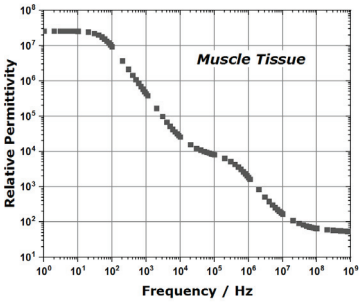

Figure 3-19
Relative permittivity of muscle tissue in the frequency region of 1 Hz – 1 GHz (Log_{10}/Log_{10} –scale)

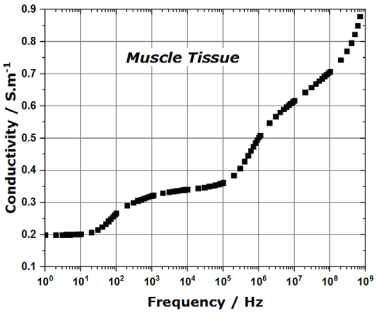

Figure 3-20
Conductivity of muscle tissue in the frequency rage of 1 Hz – 1 GHz (Log_{10} x/Lin y–scale)

TUMOUR TISSUE

Figure 3-21 displays the conductivity (S/m) for muscle tissue derived from the ITIS database, and the corresponding tumour tissue derived from actual ratios presented in *Table* 1 of Haemmerich et al. (2009) (represented by the black dots) and Eq. 2.18 (represented by solid line).[43]

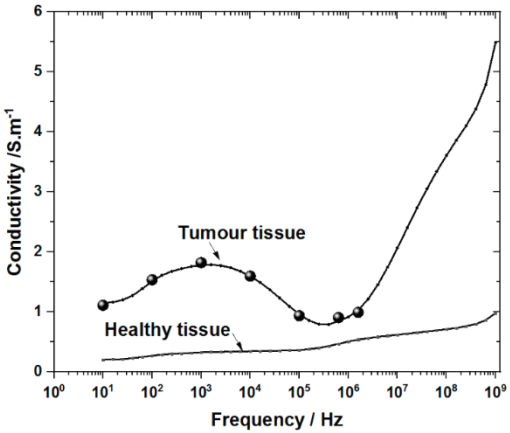

Figure 3-21
The conductivity (S/m) for healthy tissue derived from the ITIS database and tumour tissue taken from actual ratios presented in *Table 1* of Haemmerich et al. (2009) (represented by the black dots) and Eq. 2.18 (represented by the solid line).

3.5 E-FIELD, CURRENT, AND POWER DISTRIBUTION IN TISSUE

3.5.1 DISTRIBUTION OF FIELD STRENGTH AND CURRENT DENSITY

Figure 3-22 shows an estimation of the electric field-strength profile between two needle electrodes in tissues of various conductivities (0.1, 0.15, 0.2, 0.25, and 0.3 S/m) separated by 8mm with an applied voltage of 1000 V. The overall field-strength average is 870 ± 470 V/cm; and the average between electrodes is 905 ± 480 V/cm. Although the average is close to the nominal value of applied voltage divided by electrode distance, the field inhomogeneity is enormous. The E-profile and averages do not depend on the variation in tissue conductivity.[73] Eq. 3.31 estimates the current density J (A/cm^2), where the cross-section *area* of current between the needle electrodes is estimated to be 1cm^2.

$$J = \sigma \cdot E/area \qquad \text{Eq.3.31}$$

The distribution of the corresponding electric current density J-profiles, however, varies a lot with tissue conductivity, as shown in Figure 3-23.

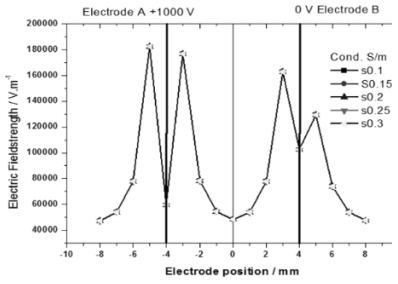

Figure 3-22
Electric field-strength (E-profile) at an applied voltage of 1000 V over 8 mm electrode spacing. Overall average, E= 870 ± 470 V/cm; average between electrodes, E= 905 ± 480 V/cm

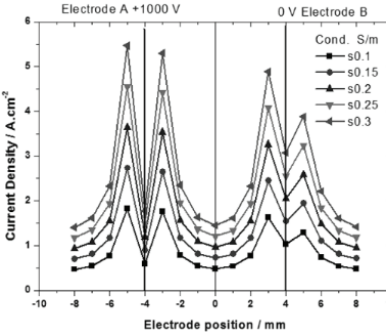

Figure 3-23
Electric current density distribution at an applied voltage of 1000 V over 8 mm electrode spacing in tissues with various conductivities of 0.1; 0.14; 0.20; 0.25; 0.30 S/m .

Figure 3-24 displays the average current density (A/cm^2) in tissues with various conductivities at 1000V between electrodes at a distance of 8mm apart. By experience, the recorded current between 2 needle electrodes in

tissue correlates well with the corresponding modelled current density (A/cm^2).

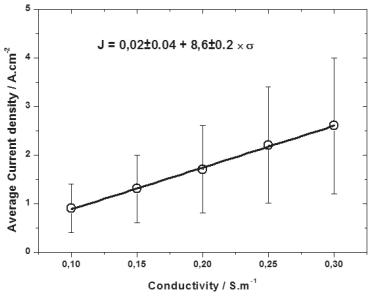

Figure 3-24
Average current density (A/cm^2) in tissues with various conductivities at 1000V between electrodes at a distance of 8 mm.

3.5.2 SPECIFIC ABSORBED ENERGY AND TEMPERATURE INCREASE

The following expression predicts the specifically absorbed energy $_sW$ in an electro-pulse treatment

$$sW = \frac{\sigma \cdot E^2}{\rho} \cdot t_p \cdot N \ [J.kg^{-1}] \qquad \text{Eq.3.32}$$

where

σ is the tissue conductivity for the tissue [S/m];
E is the electric field strength [V/m];
t_p is the pulse length [s];
N is the number of applied pulses;
ρ is the density of tissue (muscle 1060 $kg.m^{-3}$).

$$\sigma_{tafter} = \sigma_{before} \cdot R_{before}/R_{after} \qquad \text{Eq.3.33}$$

Equation 3.33 predicts the conductivity σ_{tafter} of the tissue after electro-pulse treatment with two needle electrodes separated by 8 mm[73].

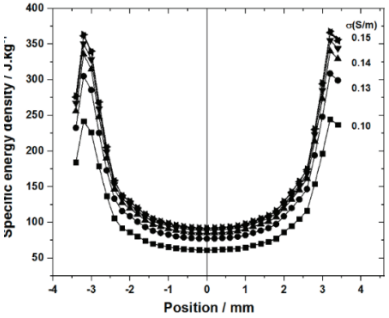

Figure 3-25
The specific absorbed energy profiles in tissues at various conductivity values achieved after each of 6 consecutive electroporation pulses of 1000 V and 100 μs pulse length[73].

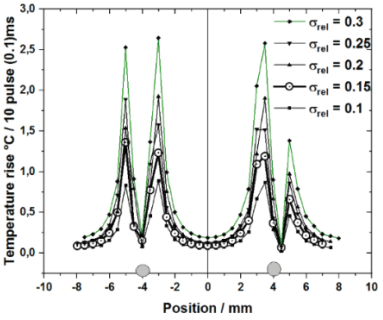

Figure 3-26
Temperature rise at different values of relative conductivity after 10 pulses (0.1ms) 1000V[73]

The thermal effect of absorbed power is heat generation through the relation:

$$sW = c \cdot \Delta T \qquad \text{Eq.3.34}$$

where

$_sW$ = the specific absorbed energy [J.kg^{-1}];
c = the heat capacity 3421 ± 460 [J.kg^{-1}.°C^{-1}];
ΔT = the change in temperature [°C].

.The value of the heat capacity of muscle tissues is 3421 ± 460 [J.kg^{-1}.°C^{-1}].[74] The temperature rises in the tissue after 10 electric 0.1ms pulses of 1000V are applied, which has been estimated using the same FEM model assuming no loss of absorbed energy (the maximum attainable temperature). At the 0.15 S/m corresponding to muscle tissue, the temperature rise does not exceed 1°C. Thus, no thermal effect is expected. In tissues with a higher conductivity of 0.3 S/m, the maximum temperature increase around the electrodes is about 2.5°C . Thus, the electroporation procedure does not induce any hyper-thermic effects.

3.5.3 CHARGE DISPLACEMENT OR CHARGE DEPOSITION

The electro-permeabilization efficiency *in vitro* depends on the applied pulse energy $\mathbf{W} \sim \mathbf{E}^2$ (where **E** is the applied field strength), while the

electro-permeabilization efficiency *in vivo* seems to depend more on the charge displacement which is equivalent to the charge density.[73]

Charge density $Q = J \cdot t_p \cdot N = \sigma \cdot E \cdot t_p \cdot N$; [As.m^{-2}] Eq. 3.35

where

σ = conductivity [S.m^{-1}];
J = current density [A.m^{-2}];
E = field strength [V.m^{-1}];
t_p = pulse length $[s]$;
N = number of pulses.

Figure 3-27 displays the result of an FEM simulation of charge density. The value of the charge density between the two needle electrodes approaches about 1.3 As/m^2 = 1.4·10^{-4}= As/cm^2= 0.14 mAs/cm^2

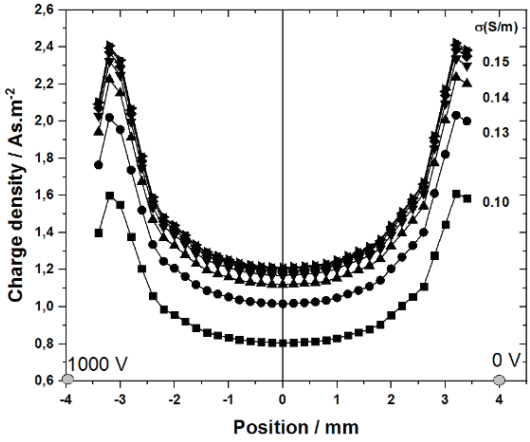

Figure 3-27
The charge density (As.m^{-2}) profiles in tissues at various conductivity values achieved after each of 6 consecutive electroporation pulses of 1000 V and 100 μs pulse length on two needle electrodes separated by 8 mm were conducted.[73]

3.6 MODELLING THE U,E,J, W DISTRIBUTIONS FOR SUCCEEDING PULSES

3.6.1 POTENTIAL DISTRIBUTION U

Figure 3-28 show the potential U(volt) profile between two needle electrodes with a diameter of 1mm inserted into muscle tissue with conductivity 0.20 S/m. The electrodes of steel with a conductivity of 6.2·10^6

S/m were separated 8 mm with an applied voltage of 1000 V at the left electrode and 0 V at the right.

At the 2nd pulse the conductivity is set to 1 S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m. Figure 3-29 displays the centre x-distribution of scalar electric potential *U*, with an applied voltage of 1000 V over two electrodes of 1 mm diameter and 8 mm electrode spacing. the conductivity is set to 1S/m in a region of 3mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m.[75]

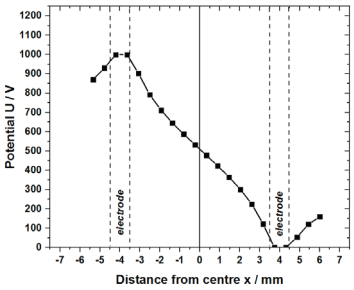

Figure 3-28
Distribution of Electric field strength E and scalar electric potential *U*, at the primary pulse with an applied voltage of 1000V over two electrodes of 0.7mm diam., and 8mm electrode spacing.

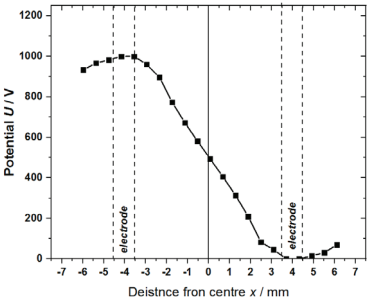

Figure 3-29
Centre x-distribution of scalar electric potential *U*, at the 2nd pulse with an applied voltage of 1000V over two electrodes of 1 mm diameter and 8 mm electrode spacing. The conductivity is set to 1S/m in a region of 3mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2S/m.

3.6.2 ELECTRIC FIELD-STRENGTH DISTRIBUTION E

The upper figure 3-30 shows the distribution of Electric field-strength **E,** modelled between two needle electrodes with a diameter of 1 mm inserted into muscle tissue with conductivity 0.20 S/m. The electrodes of steel with a conductivity of $6.2 \cdot 10^6$ S/m were separated 8 mm with an applied voltage of 1000 V at the left electrode and 0V at the right.

The lower figure 3-30 displays the profile of electric field-strength distribution along a horizontal line through the electrodes. The average electric field in the region of ± 2 mm from the centre estimated by modelling with various tissue conductivities shown in Figure 3-5 is about 1050 V/cm

independent of conductivity.

The upper figure 3-31 shows the distribution of electric field strength E at the 2nd pulse with an applied voltage of 1000 V over two electrodes of 1 mm diameter and 8 mm electrode spacing. The conductivity is set to 1 S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m.

The lower figure 3-31 displays the profile of the electric field strength ***E*** along the centre ***x***-axis, with an applied voltage of 1000 V over two electrodes of 1 mm diam., and 8 mm electrode spacing. The conductivity in region of 3 mm diameter around the electrode is increased to 1 S/m due to the effect of the first pulse, while the rest of tissue has a conductivity of 0.2 S/m. The average field strength along a horizontal line in the region ± 2 mm between the electrodes is about 1750 V/cm, compared to 1150 V/cm in the first pulse.

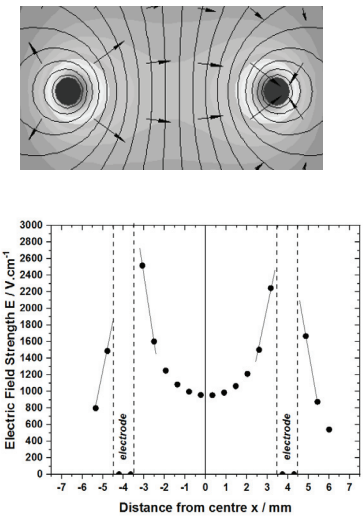

Figure 3-30
Distribution of electric field strength E, at the primary pulse with an applied voltage of 1000 V over two electrodes of 1mm diam. and 8 mm electrode spacing in muscle tissue with conductivity 0.2 S/m.

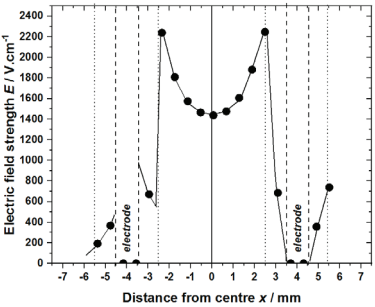

Figure 3-31
Distribution of electric field strength E, at the 2nd pulse with an applied voltage of 1000 V over two electrodes of 1mm diameter and 8 mm electrode spacing. The conductivity is set to 1 S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m.

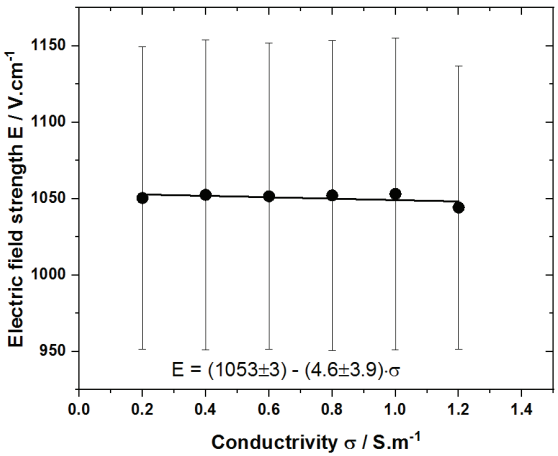

Figure 3-32
The average Field strength at the primary pulse in tissue of various conductivity in the central region of ±2 mm between two electrodes of 1mm diam., and 8mm electrode spacing with an applied voltage between the electrodes of 1000V.

3.6.3 CURRENT DENSITY DISTRIBUTION J OF THE PRIMARY PULSE

Figure 3-33 shows the average electric current density in the region of ±2mm from the centre estimated by modelling with various tissue conductivities σ, increase linearly with conductivity. The current density expressed in A.cm^{-2} corresponds approximately to the recorded current between the electrodes.

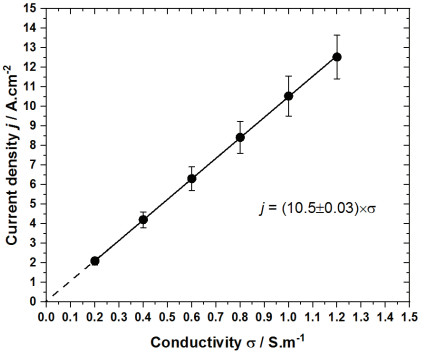

Figure 3-33
The average current density j in tissue of various conductivities in the region of ± 2mm between two electrodes of 1 mm diameter, and 8 mm electrode spacing at an applied voltage between the electrodes of 1000 V.

$$I \approx 10 \cdot \sigma$$

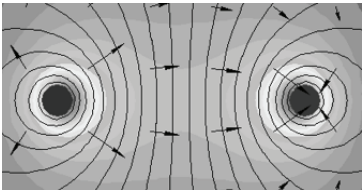

Figure 3-34
Distribution of current density j with an applied voltage of 1000 V over two electrodes of 1mm diam. and 8mm electrode spacing in muscle tissue with conductivity 0.20S/m.
The average current distribution in the region ± 2mm between the electrodes is about 2A.cm^{-2}.

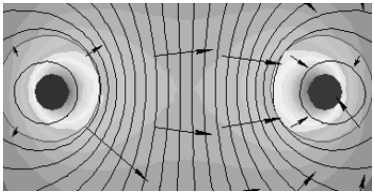

Figure 3-35
Distribution of electric current distribution *j* at the 2nd pulse with an applied voltage of 1000V over two electrodes of 1mm diameter and 8mm electrode spacing. The conductivity is set to 1S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2S/m.
The average current density in the region ± 2 mm between the electrodes is about 3.5A.cm^{-2}

Figure 3-34 shows the distribution of electric current density **j** modelled between two needle electrodes with a diameter of 1 mm inserted in muscle tissue with conductivity 0.20S/m. The electrodes of steel with a conductivity of $6.2 \cdot 10^6$ S/m were separated 8mm with an applied voltage of 1000 V at the left electrode and 0V at the right.

Figure 3-35 shows the distribution of electric current distribution ***j*** at the 2nd pulse with an applied voltage of 1000 V over two electrodes of 1 mm diameter and 8 mm electrode spacing. The conductivity is set to 1 S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m. The average is about 3.5 A.cm^{-2}, compared to 2 A.cm^{-2} in the first pulse. Thus in order to keep the current density at the level of the first pulse the applied voltage amplitude of the second pulse should be reduced.

3.6.4 DISTRIBUTION OF ABSORBED POWER DENSITY P OF THE PRIMARY PULSE

Figure 3-36 shows the absorbed power density P_v (W/m^3) between two needle electrodes with a diameter of 1 mm inserted into muscle tissue with conductivity 0.20 S/m. The electrodes of steel with a conductivity of $6.2 \cdot 10^6$

S/m were separated 8 mm with an applied voltage of 1000 V at the left electrode and 0 V at the right.

The average absorbed power density along a horizontal line in the region ± 2 mm between the electrodes is about $2.22 \cdot 10^9$ W.m^{-3}, which corresponds to $2.22 \cdot 10^6$ W/kg.

For a 100 μs long pulse of 1000 V/cm amplitude the absorbed energy density is $2.22 \cdot 10^2$ J/kg = 0.22 J/g = 0.22 Mir[*)].

[*)] The unit for absorbed dose in radiation therapy is named "gray", (1 Gy = 1 J/kg) to honour Louis Harold Gray.
The unit for absorbed dose in electro chemo therapy is here suggested to be named "mir", (1 Mr = 1 J/g), to honour Lluis M Mir for his great contributions to the field of electrochemotherapy.

Figure 3-37 shows the distribution of **absorbed power density P_V** distribution at the 2nd pulse with an applied voltage of 1000 V over two electrodes of 1 mm diameter and 8 mm electrode spacing. The conductivity is set to 1 S/m in a region of 3 mm outer diameter around the electrode while the rest of tissue has a conductivity of 0.2 S/m.

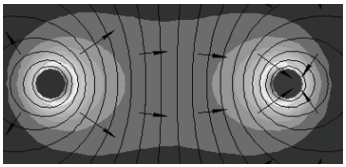

Figure 3-36
Distribution of absorbed power density P_v W/m^3 with an applied voltage of 1000 V over two electrodes of 1mm diam. and 8mm electrode spacing in muscle tissue with conductivity 0.20S/m.

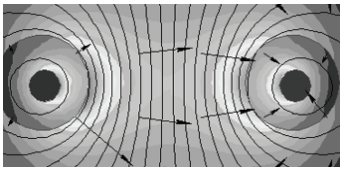

Figure 3-37
Distribution of absorbed power density P_V distribution at the 2nd pulse with an applied voltage of 1000V over two electrodes of 1mm diam. and 8 mm electrode spacing and a region of 3 mm diameter around the electrode with the conductivity of 1S/m while the rest of tissue has a conductivity of 0.2S/m.

At the second pulse the average **absorbed power density** in the region ± 2 mm between the electrodes is about 0.63 J/g compared to 0.22 J/g at the first pulse.

3.6.5 SPECIFIC ABSORBED ENERGY AND TEMPERATURE INCREASE

The specific absorbed energy SAE is calculated from the following expression

$$SAE = \frac{\sigma \cdot E^2}{\rho} \cdot t_p \cdot N \ [J.kg^{-1}] \qquad \text{Eq. 3.36}$$

where

σ is the tissue conductivity for the tissue [S/m]
E is the electric field strength [V/m]
t_p is the pulse length [s]
N is the number of applied pulses
ρ is the density of tissue (muscle 1060) kg.m^{-3}

The following equation predicts the conductivity of the tissue after electroporation

$$\sigma_{after} = \sigma_{before} \cdot R_{before}/R_{after} \qquad \text{Eq. 3.37}$$

The average specific absorbed energy in tissue of various conductivity of a single 100 μs long pulse of 1000 V amplitude varies with the conductivity in the region of ± 2 mm between two electrodes of 1 mm diam., and 8 mm electrode spacing of the medium as equation Eq. 3.38.

$$\text{SAE} = (1.11 \pm 0.006)\times\sigma; \quad [\text{J/g}] \qquad \text{Eq. 3.38}$$
$$\text{R-Square (COD)} = 0.9998$$

The value for the heat capacity of muscle issues is 3421 ± 460 [J.kg^{-1}.°C^{-1}] .[74] The temperature rise in the tissue after in tissue of various conductivity of a single 100 μs long pulse of 1000 V amplitude varies with the conductivity in the region of ± 2 mm between two electrodes of 1 mm diam., and 8 mm electrode spacing of the medium is given in the equation:

$$\Delta T = (0.33 \pm 0.05)\times\sigma; \ [°C] \qquad \text{Eq. 3.39}$$

Thus the electro pulse therapy procedure does not do not induce any significant hyper-thermic effects.

3.6.6 DISCUSSIONS

The extensively increase of the electric-field in the centre of the target volume could be avoided by decreasing the amplitude of the second and following pulses in the pulse train. Figure 3-38 shows an example of a pulse sequence with exponentially decreasing pulse amplitude with a time constant of 1 ms. A pulse sequence with exponentially decreasing pulse amplitude an excellent therapy efficiency of adenocarcinoma implanted in rat liver with a high degree of immune response, resulting in anti-metastatic effect.[76]

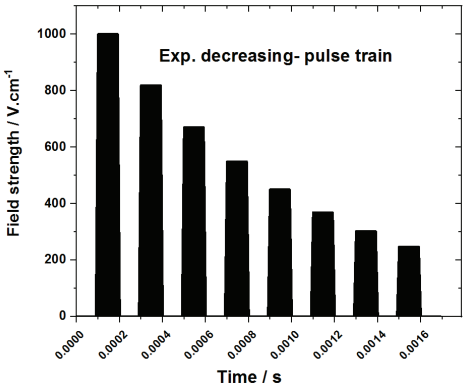

Figure 3-38
Pulse train of Exponentially decreasing E-field that could preferentially apply to Electro-pulse therapy.

CHAPTER IV

GAMMA CAMERA STUDIES OF ELECTRO-PULSE ENHANCED DRUG UPTAKE

4.1 GAMMA CAMERA STUDIES OF BLEOMYCIN UPTAKE

Per Engström (1998) in his thesis work studied the uptake and retention of intravenously administered Bleomycin labelled with radioactive Indium-111. After treatment with electric pulses, gamma camera measurements verified the distribution of ^{111}In-Bleomycin in Fischer 344 rats with subcutaneously implanted N32 glioma tumours.[77,78,79,80]

Figure 4-1 shows a gamma-camera image taken 2 days after intravenous administration of ^{111}In-Bleomycin in non-treated rats. There is no uptake in the area of the implanted tumour, while a high accumulation of ^{111}In appears in the kidneys.[79,78]

Figure 4-2 shows the gamma camera images of ^{111}In-Bleomycin accumulation 3 days after electro-pulse treatment. ^{111}In- Bleomycin gradually disappears from circulation, while it stays in the electro-pulse treated tumour and the kidneys for several days.[80]

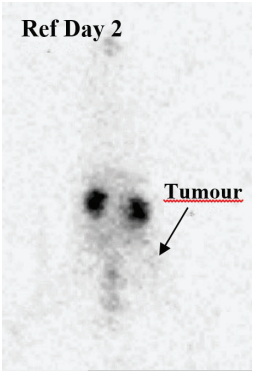

Figure 4-1
Gamma camera image of a non-treated rat 2 days after intravenous administration of ^{111}In- Bleomycin, showing uptake in the kidneys while no accumulation appears in the area of the transplanted tumour.[80]

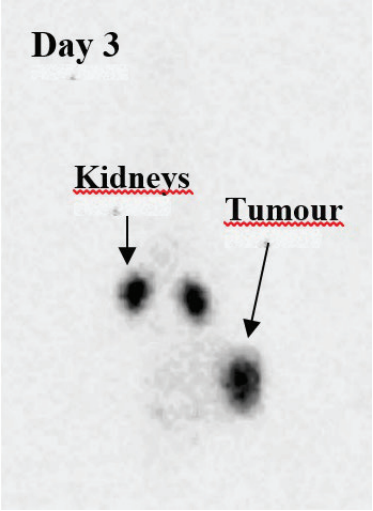

Figure 4-2
Gamma camera image showing ^{111}In-Bleomycin accumulation 3 days after electro-pulse treatment of a tumour .[80]

4.2 ELECTRO-ENHANCED UPTAKE OF ^{99m}Tc-DTPA

4.2.1 UPTAKE MEASUREMENTS OF ^{99m}Tc-DTPA

^{99m}Tc-DTPA (diethylene-triamine-penta-acetic acid) is a common, clinically used radiopharmaceutical which shows several pharmacokinetic similarities with Bleomycin.[81] Intramuscular injection (i.m.) of 150MBq ^{99m}Tc-DTPA in several fractions of 50 μl each, at 1-minute intervals in the shoulder of a rat, created a depot of ^{99m}Tc-DTPA for gradual uptake in the circulation in order to explore the enhanced uptake with a gamma camera after electric pulse treatment.[56,58]

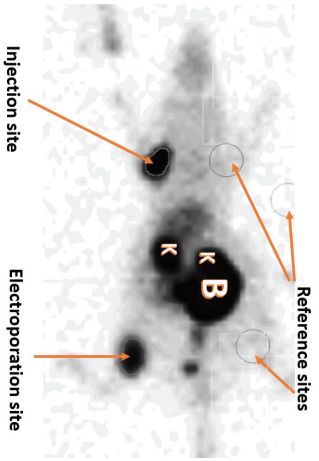

Figure 4-3
GK-image 6 hours after electro-pulse treatment of a rat with 12 pulses of 800 V/cm and 0.25 ms. K= kidney, B= bladder.

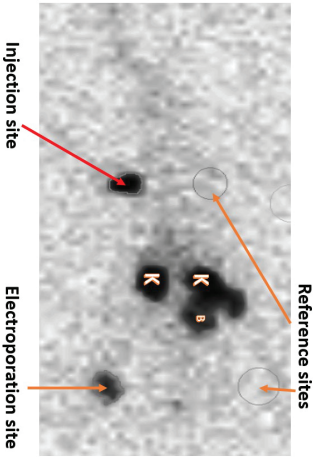

Figure 4-4
GK-image 24 hours after electro-pulse treatment of the same animal as in Fig 4-3. K= kidney, B= bladder.

Figure 4-3 shows a typical gamma camera (GK) image of the distribution of radioactivity at 6 and 24 hours after electroporation in the thigh muscle of a rat. The administration site on the shoulder appears as a dark spot, and the uptake in the electric pulse-treated region on the thigh shows as a dark spot. The corresponding areas at the untreated opposite sides of the rat represent the reference area for extracellular activity. Kidney (K) and bladder (B), showing urine activity, are visualized as dark areas in the image.

In the evaluation of the activity uptake, a line profile in the gamma camera images crosses the treated region within the "full width at half maximum" (FWHM). This measure defined the diameter of the circular region of interest (ROI) used for the evaluation of the drug uptake-ratio.

During the year 2000, the author performed a gamma camera imaging study of how 2, 4, 6 or 12 applied electric pulses of different field-strengths (600; 800; 1000: 1200: V/cm and pulse length 100 or 500 μs) influence the uptake of the radiolabelled pharmaceutical ^{99m}Tc-DTPA in rat muscular tissue. Figure 4-3 shows a typical gamma-camera image six hours after electro-pulse treatment in the thigh muscle of a rat, and Figure 4-4 the corresponding image after 24 hours.

Table 4-1 displays the results for each rat: the applied field strength (V/cm); pulse-length (μs); number of applied pulses N; and the uptake-ratio

of ^{99m}Tc-DTPA in the target region after six hours (UR_{6h}) and after 24 hours (UR_{24h}) respectively.

Table 4-1

Applied field strength (V/cm), pulse-length (µs), number of applied pulses N, and the uptake-ratios of $^{99}Tc^{m}$-DTPA in the target region after six hours (UR_{6h}) and 24 hours (UR_{24h}).

E (V/cm)	t (µs)	N	TcUR 6h	TcUR 24h
800	500	8	8.27	3.57
800	500	8	5.87	4.04
800	500	8	6.45	4.08
225	500	8	1.74	
225	500	8	1.01	
225	500	8	1.14	
400	500	8	1.47	2.43
400	500	8	3.25	4.05
400	500	8	5.36	2.79
1200	500	8	9.07	6.13
1200	500	8	9.01	3.14
1200	500	8	8.45	4.97
1800	500	8	10.12	
1800	500	8	7.82	
400	500	8	6.61	
1500	500	8	7.25	4.88
800	500	8	4.85	3.69
Table continued				
800	500	8	5.38	3.83
800	500	8	6.46	2.72
1500	500	8	7.76	6.32
1500	500	8	7.85	6.14
1200	500	8	9.01	9.07
1200	500	8	5.81	4.7
1200	500	8	5.53	4.22
1200	500	6	8.92	7.15
1200	500	6	4.41	2.79
1200	500	6	7.73	4.12
1200	500	10	8.66	8.01
1200	500	10	7.64	6.48
1200	500	10	11.4	5.82
1200	500	12	12.84	14.17
1200	500	12	11.23	5.61
1200	500	12	7.59	8.05
1200	500	4	10.35	8.36
1200	500	4	7.57	8.93
1200	500	2	3.34	3.22

1200	500	2	2.29	1.53
1200	500	2	2.92	2.52
1000	500	12	10.72	18.13
1000	500	12	14.52	6.74
1000	500	12	10.14	13.05
800	500	12	13.31	12.11
800	500	12	11.2	9.32
800	500	12	10.25	9.3
600	500	12	9.21	7.13
600	500	12	5.76	5.88
800	250	12	12.93	17.23
800	250	12	11.1	18.9
800	250	12	12.28	21.78
1200	500	4	13.49	14.81
1200	500	4	7.59	17.49
1200	500	4	6.18	3.02
1000	100	6	1.45	1.61
1000	100	6	3.58	4.44
1000	100	6	3.26	11.59
1000	100	12	9.72	5.77
1000	100	12	10.63	12.84
1000	100	12	6.66	11.99

Statistical analysis of the data applies multivariate data processing methods, such as principal component analysis (PCA).

Modelling of the data uses the method of projection to latent structures (PLS), also called partial least square regression (PLSR). Herman Wold introduced the practice of this method,[82] and his son, Svante Wold, a chemist (and in 1963, my comrade in military service), developed its application for chemometrics.[83] According to him, the projection to latent structures should be the correct name of the technique.[84,85] These methods are nowadays commonly used in chemometrics, bio-pharmacology and related areas.

Table 4-2 shows the correlation matrix of the uptake ratios with field-strength E(V/cm), pulse-length PL(ms), and the number of pulses:

Table 4-2
Correlation matrix with E, PL and N as descriptor variables, and with UR_{6h}, and UR_{24h} as depending variables.

Variables	E V/cm	PL ms	N pulses	UR6h	UR24h
E V/cm	**1.000**	0.305	-0.591	-0.252	-0.356
PL ms	0.305	**1.000**	-0.163	0.172	-0.197
N pulses	-0.591	0.163	**1.000**	0.616	0.434
UR_{6h}	-0.252	0.172	0.616	**1.000**	0.612
UR_{24h}	-0.356	-0.197	0.434	0.612	**1.000**

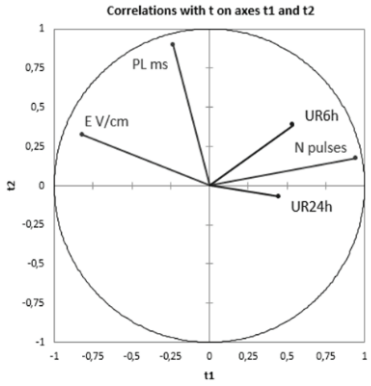

Figure 4-5
The plot of the correlation of variables with the t1 and t2 components of PLS modelling.

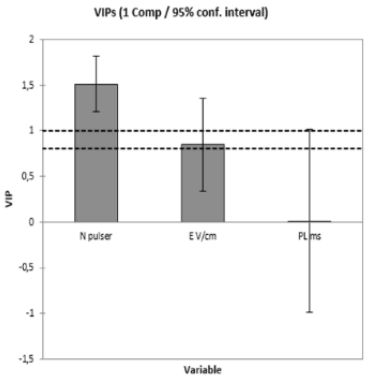

Figure 4-6
The *variable importance of projections* (VIP) of the experimental variables used in the modelling of the projection of latent variables.

Figure 4-5 displays the PLS score values t2 as plotted against the values t1 for all variables. The uptake rations show a strong correlation with the number of pulses, but are independent of pulse-length, and.inversely correlated to the applied pulse length. Figure 4-6 also shows that the number of pulses is the most important variable of projection to latent structures (VIP), followed by the field strength

Table 4-3 shows the parameters of the PLS model equations (Eq. 4.1 and Eq. 4.2) which predict the value of the uptake-ratios at 6 and 24 hours respectively. It is interesting that the parameter for the applied field strength is negative at both the 6 and 24-hour uptake-ratio, which indicates the benefit of decreasing field-strength in clinical applications.

Figures 4-7 and 4-8 display the linear relationships of the uptake ratios predicted according to the PLS modelling equations as opposed to the measured values.

Table 4-3
PLS Model parameters of UR

Variable	UR_{6h}	UR_{24h}
Intercept	1.709	12.342
E V/cm	$-1.32 \cdot 10^{-3}$	$-5.87 \cdot 10^{-3}$
PL µs	$7.67 \cdot 10^{-3}$	$-2.01 \cdot 10^{-3}$
N pulses	0.571	0.409
R^2	0.44	0.21

Equations of the model:

$$UR_{6h} = 1.709 - 1.32 \cdot 10^{-3} \times E + 7.67 \cdot 10^{-3} \times PL + 0.571 \times N \quad \text{Eq. 4.1}$$

$$UR_{24h} = 12.342 - 5.87 \cdot 10^{-3} \times E - 2.01 \cdot 10^{-3} \times PL + 0.409 \times N \quad \text{Eq. 4.2}$$

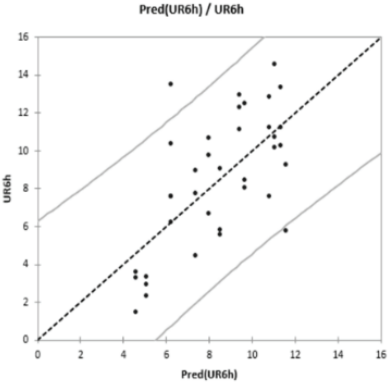

Figure 4-7
The regression between the experimental and predicted uptake ratios 6 hours after EP

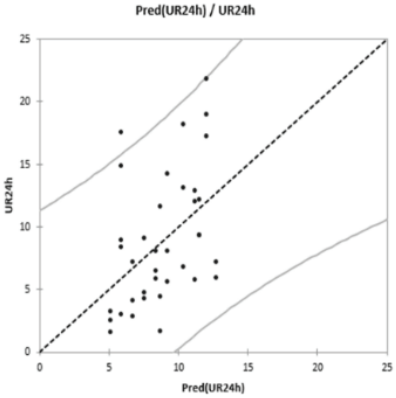

Figure 4-8
The regression between the experimental and predicted uptake ratios 24 hours after EP

4.2.2. EXPLORATION OF CONDUCTIVITY CHANGE AND LOSS CHANGE INDEX

Conductance and phase angle θ measurements were performed at 2 and 20kHz in Fischer 344 rats with two needle electrodes separated by 8mm inserted in the right posterior thigh muscle before and after applying the EP treatment pulse. The first two measurements established the reference level R_{before} and $\tan(\theta_{before})$, before the measurements were performed between each N_p consecutive pulse (Np = 2, 4, 6, 10, 12) of field strength amplitude E = 600, 800, 1000,1200 V/cm, and pulse-length 100, 250, 500 μs applied at 1s intervals. To study the relaxation of the electroporation, the conductance measurements were continued 15 times after the last pulse in 1s intervals.

Table 4-4 shows the model parameters of PLS modelling the resistivity change index (Eq. 3.28), and the loss change index (Eq. 3.23) recorded at 2 and 20kHz as dependent variables, and with field strength amplitude E, pulse length PL and number of pulses Np as descriptor variables.

Table 4-4
Model parameters of PLS modelling RCI and LCI.

Variable	RCI_{2kHz}	RCI_{20kHz}	LCI_{2kHz}	LCI_{20kHz}
Intercept	-0.226	-0.288	0.114	-0.280
E V/cm	$3.76 \cdot 10^{-4}$	$3.85 \cdot 10^{-4}$	$1.20 \cdot 10^{-4}$	$2.65 \cdot 10^{-4}$
PL μs	$2.75 \cdot 10^{-4}$	$2.57 \cdot 10^{-4}$	$2.49 \cdot 10^{-4}$	$3.03 \cdot 10^{-4}$
N pulses	0.024	0.023	0.012	0.034
R^2	0.453	0.392	0.491	0.723

Correlation of the first and second principal latent component of the various variables shown in Figure 4-9 indicates that the resistivity and phase variables are closely correlated to the pulse length and number of applied pulses. The field-strength, however, indicates a negative correlation to the number of pulses, and has no relationship with the other parameters.

Figure 4-10 also shows that “the number of pulses” is the variable of most importance to the projection of latentstructures (VIP), followed by the pulse length. However, the field strength seems to be of less importance.

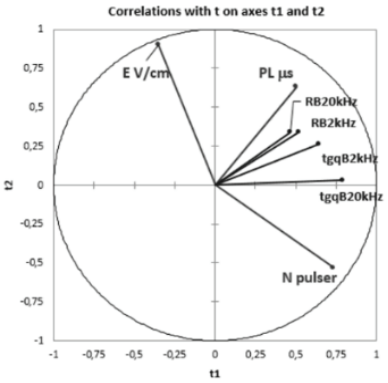

Figure 4-9
The correlation plot of the first and second principal latent components of the various variables.

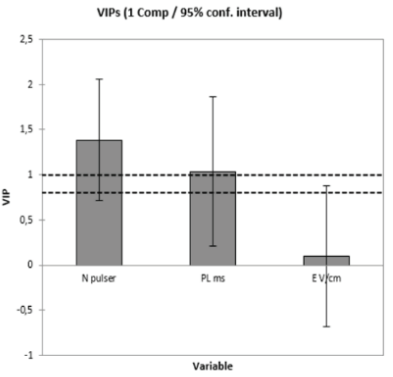

Figure 4-10
Variable importance in the 1st projection (VIP) with the largest variance.

The best fitting to the PLS model was found for LCI recorded at 20 kHz, for which the following equations of the PLS model were achieved:

Loss Change Index $LCI_{20\ kHz}$:

$$LCI_{20kHz} = -0.280 + 0.034 \times N + 3.03 \cdot 10^{-4} \times PL + 2.65 \cdot 10^{-4} \times E \qquad \text{Eq. 4.3}$$

Table 4-5 shows the current density J A/cm^2 (Eq. 3.3) estimated by Eq. 3.31 and specific absorbed energy W calculated from the Eq. 3.32 from the values of E, PL, and N from a series of rat experiments.

Table 4-5
The current density J A.cm^{-2} and specific absorbed energy W J.kg^{-1} with E, PL, and N as predictor variables.

Rat ID	E V/cm	PL µs	N pulses	J A.cm^{-2}	W J.kg^{-1}
1	1200	500	2	14.47	3276
2	1200	500	2	16.17	3660
3	1200	500	2	15.32	3547
4	1200	500	4	15.50	7018
5	1200	500	4	25.00	11321
6	1200	500	4	20.69	9370
7	1200	500	4	16.89	7647
8	1200	500	4	17.07	7732
9	1200	500	4	29.38	13306
10	1200	500	10	15.67	17735
11	1200	500	10	18.95	21448
12	1200	500	10	23.29	26364
13	1200	500	12	32.19	43725
14	1200	500	12	37.57	51039
16	1000	100	6	2.74	1549
17	1000	100	6	2.22	1256
18	1000	100	6	2.34	1326
19	1000	100	12	2.32	2632
20	1000	100	12	2.63	2983
21	1000	100	12	3.12	3529
22	1000	500	12	3.34	21165
23	1000	500	12	3.82	15574
24	1000	500	12	3.16	17881
25	800	500	12	2.15	9754
26	800	500	12	2.28	10303
28	800	250	12	1.91	4327
29	800	250	12	3.11	7034
30	800	250	12	2.66	6029
31	600	500	12	1.64	5580
32	600	500	12	1.44	4899
15 *outlayer*	1200	500	12	258.15	45482

Figure 4-11 displays the correlation of t1 and t2 PLS modelling scores with J and W acting as dependent variables and field strength amplitude E, pulse length PL and number of pulses Np as descriptor variables. The plot in Figure 4-11 indicates that the current density J and specific absorbed energy W correlate to the applied field-strength E and pulse length. Figure 4-12 shows the applied field-strength to be the most *important variable in the projection* (VIP) followed by pulse-length. The number of pulses seems to be less critical.

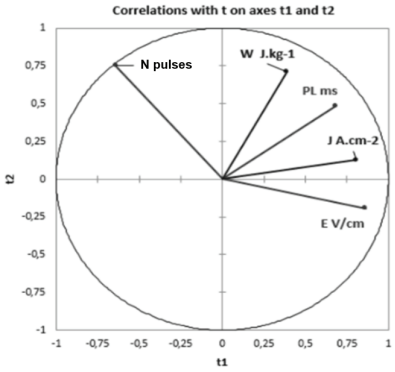

Figure 4-11
A correlation plot of the first and second principal latent components of the various variables

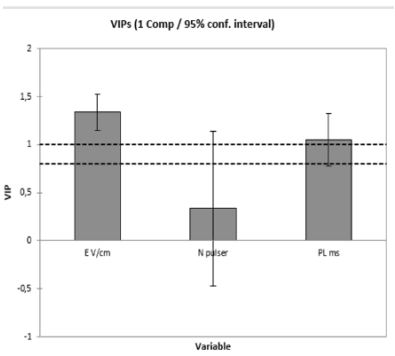

Figure 4-12
Variable importance in the 1st projection (VIP) with the most substantial variance

With current density J and specific absorbed energy W, as dependent variables, and applied field strength, pulse length and number of pulses as descriptor variables, a PLSR modelling resulted in the following model equations:

$$J\,[A.cm^{-2}] = -45.2 + 0.043 \times E + 0.022 \times PL + 0.398 \times N \qquad \text{Eq.4.4}$$

$$W[J.kg^{-1}] = -71791 + 47.9 \times E + 28.6 \times PL + 2616 \times N \qquad \text{Eq. 4.5}$$

4.2.3 EXPLORATION OF CONDUCTIVITY RELAXATION

After the applied electro-permeabilization pulse, the conductivity started to decrease and approach a plateau value. The fraction of the plateau value relative to the conductivity recorded after the last pulse is a measure of the fraction of reversible electropermeabilized cells. This value is of importance for the long-term transfer of exogenous substances to the cell and outflow of immunogenic material from the cell. Figure 4-13 displays the results of PLS modelling, with the fraction of reversible relaxation F_{rev} evaluated by Eq. 3.30 using the relaxation curves and the mean relaxation time T for each rat fitted to a single exponential decay as dependent variables and field strength amplitude E, pulse length PL and number of pulses Np as descriptor variables.

The plot in Figure 4-13 indicates that a fraction of reversible relaxation F_{rev} correlates positively to the applied field-strength E and negatively to the number of applied pulses. Figure 4-14 shows the applied field-strength to be the most important variable in the projection (VIP), followed by the number of pulses. The pulse length seems to be less critical.

The PLS model parameters given in Table 4-6 show a negative relationship to the applied field strength and pulse length, while the fraction of reversible relaxation F_{rev} shows a positive relationship.

$$T\ [s^{-1}]=6.056 - 1.82\cdot10^{-3}\times E - 1.18\cdot10^{-3}\times PL + 0.065\times N; \quad R^2 = 0.30 \qquad \text{Eq. 4.6}$$

$$F_{rev} = -0.257 + 5.07\cdot10^{-4}\times E + 3.28\cdot10^{-4}\times PL - 0.018\times N; \quad R^2 = 0.73 \qquad \text{Eq. 4.7}$$

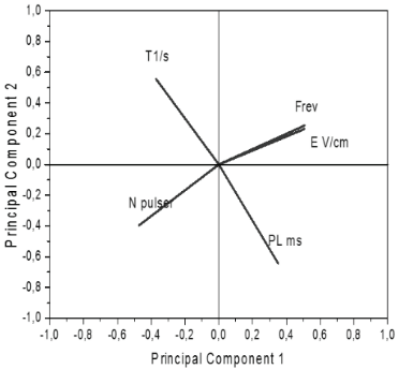

Figure 4-13
A correlation plot of the first and second principal latent components of the various variables

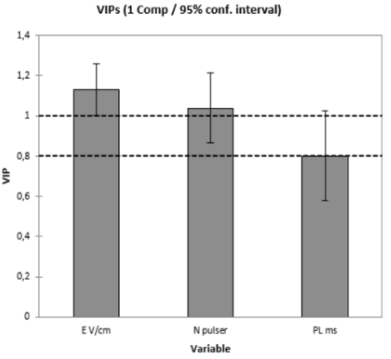

Figure 4-14
Variable importance in the projection (VIP) of the variables with the most substantial variance

4.3 CONCLUSION

The parameters with the most regression to the drug uptake ratio 6 and 24 hours after electroporation are LCI_{20kHz}; LCI_{2kHz}; W $J.kg^{-1}$; and RCI_{2kHz}.[56,58] Thus, the most optimal scenario to predict the outcome of the electro-chemotherapy session (i.e., to achieve the highest uptake ratio of Bleomycin) would be to use LCI_{20kHz} and LCI_{2kHz} as descriptors beside the parameters E, PL and N. Figure 4-15 displays the variable importance in the projection (VIP):of PLS modelling with all descriptor variables. Figure 4-16 displays the variable importance in the projection (VIP) of PLS modelling with only LCI and the number of pulses as descriptor variables.

PLS modelling with all five descriptor variables (LCI_{20kHz};LCI_{2kHz}, E kV/cm, PL ms, and N) resulted in the following equation with the coefficients summarized in Table 4-6:

$$UR_{6h} = 2.22 - 1.85\times E - 0.22\times PL + 0.22\times N + 9.38\times LCI_{2kHz} + 6.32 x LCI_{20kHz} \quad \text{Eq.4.8}$$

$$UR_{24h} = 3.37 - 1.61\times E - 0.19\times PL + 0.19\times N + 8.13\times LCI_{2kHz} + 5.48 x LCI_{20kHz} \quad \text{Eq.4.9}$$

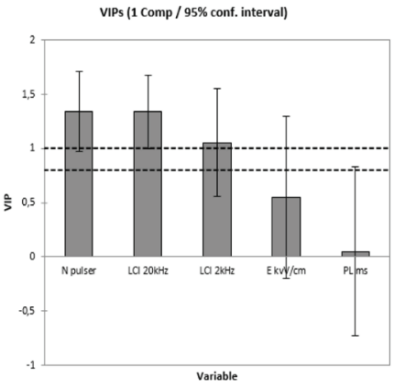

Figure 4-15
Variable importance in the projection (VIP) of all descriptor variables

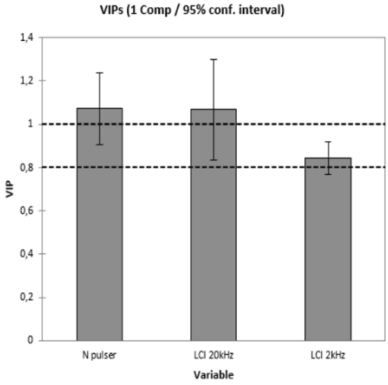

Figure 4-16
Variable importance in the projection (VIP) of LCI and number of pulses as the descriptors

Surprisingly the coefficient for the E-field becomes negative, which means that the uptake decrease by increasing the field strength. It also indicates that the large inhomogeneity of the field is of less importance.

Table 4-6
Model parameters of PLS modelling uptake ratio

Variable	UR_{6h}	UR_{24h}
Intercept	2.22	3.37
E kV/cm	-1.85	-1.61
PL ms	-0.22	-0.19
N pulses	0.22	0.19
LCI_{2kHz}	9.38	8.13
LCI_{20kHz}	6.32	5.48
R^2	0.46	0.16

Table 4-7 displays the modelling results achieved through PLS modelling by omitting the E-field and pulse length as descriptor variables.

Table 4-7
Model parameters of PLS modelling

Variable	UR_{6h}	UR_{24h}
Intercept	-0.51	-1.46
N pulses	0.24	0.20
LCI_{2kHz}	10.17	8.34
LCI_{20kHz}	6.82	5.60
R^2	0.47	0.14

$$UR_{6h} = -0.51 + 0.24\times N + 10.17\times LCI_{2kHz} + 6.82 x LCI_{20kHz} \quad \text{Eq.4.10}$$

$$UR_{24h} = -1.46 + 0.20\times N + 9.34\times LCI_{2kHz} + 5.60 x LCI_{20kHz} \quad \text{Eq.4.11}$$

CHAPTER V

PRECLINICAL STUDIES OF ELECTRO-PULSE ENHANCED THERAPY

5.1 PRECLINICAL *EpEChT* STUDIES WITH BLEOMYCIN

5.1.1 INTRODUCTION

Okino and Mohri reported the first preclinical study using the electroporation effect for enhanced transport of the anticancer drug Bleomycin across the cell membrane in 1987.[26,27] The first clinical trial using Bleomycin and electric pulse treatment (known as "electro-chemotherapy" (ECT)) was performed on head and neck tumours by Mir and colleagues in France in 1991.[86,87,88,89] Following clinical trials, this method has, as of 2018, been used to treat more than 1500 patients with either malignant melanoma, Kaposi's sarcoma, breast cancer, squamous cell carcinoma and basal cell carcinoma. The clinical results reviewed and analysed in the following chapter are very challenging. These results of extensive preclinical studies indicate enhanced therapeutic effects as a consequence of combining pulsed electric field treatment with chemotherapy, immunotherapy, and radiation therapy regimes.

Electro-pulse-enhanced chemotherapy (*EpEChT*) investigations use many different tumour models in mainly small mammals such as rats and mice. Because of their accessibility and practicality, coetaneous and subcutaneous malignancies constitute the major part of these studies. The pulse-enhanced protocols used clinically initially were derived from in vitro studies. Most of these studies use square wave pulses or exponentially decaying pulses.[78,90,91]

The amplitudes of the applied electric fields vary from 800 to 1500 V/cm. The pulse length used in square wave protocols is usually about 0.1ms, and in exponential pulse protocols, the mean decay time is about 1ms. The number of pulses applied is typically between 6 and 12.

Transmembrane breakdown of cells *in vivo*, as well as *in vitro*, occurs only when the external field exceeds a specific threshold value. This

threshold, however, is more difficult to define *in vivo* than *in vitro*. Drug and gene delivery by in vivo electro-permeabilization of muscle cells occurs at a field strength of 100-200V/cm and pulse lengths of 20-100ms.[92,93,31] The electro-permeabilization efficiency also depends on the size of cells and the dielectric properties of the tissue. One remarkable observation from all the animal studies of electro-pulse enhanced chemotherapy is the overall favourable treatment efficiency, regardless of the tumour model.

Electro-pulse -enhanced chemotherapy has been performed on the following tumour models:

- mammary tumours,[94]
- melanoma,[95,30,96]
- hepatic tumours from colorectal cancer,[37,76]
- hepatomas,[36]
- subcutaneous and orthotopic pancreatic adenocarcinoma.[38]
- soft tissue sarcomas,[33,97,98]
- bladder cancer,[99,100]
- subcutaneous and intracerebral gliomas,[78,34]

Electropermeabilization has also been employed to enhance the intratumoural uptake of Boron-compounds in Boron neutron capture therapy of malignant gliomas.[101]

Electro-pulse-enhanced chemotherapy has hitherto mainly been used as single-treatment cancer therapy. However, treatments performed once a week on three occasions on mice with B16 melanoma, a tumour with a very high recurrence rate, markedly improved the response rate and increased the survival time, although the cure rate was low.[102,103]

5.1.2 ELECTRO-PULSE ENHANCED TREATMENT OF RAT GLIOMA

The first intracerebral electro-pulse-enhanced chemotherapy was performed in 7 rats with RG2 glioma cells inoculated in the brain and electric pulses delivered through two acupuncture needles inserted 5mm into the parenchyma on each side of a tumour.[34] Bleomycin was administered intravenously before electric pulse delivery. Two complete remissions were recorded and a significantly prolonged survival time of 200% was achieved for the seven treated animals compared to all other groups.

Bleomycin reaches, and to some extent accumulates in, glioma and meningioma after intravenous injection, via the disrupted blood-brain barrier.[104,105,106,107,108] A study of intracerebral electro-pulse chemotherapy treatment performed with radioactive ^{111}In-labelled Bleomycin resulted in several cases of complete remission of N32 tumours.[79]

5.1.3 ELECTRO-PULSE ENHANCED TREATMENT OF RAT LIVER TUMOURS

Since 1997, four studies have reported on electro-pulse-enhanced chemotherapy of tumours treated in the liver, of which two have been colorectal cancer.[37,36,109,110]. The treatment protocol and outcome of these studies are summarised in Table 5-1 and compared to the one described in 2001 by Engström and collaborators.[76] In all studies, except one, Bleomycin was applied intralesionally to a tumour.[110] Generally, the studies used low doses of Bleomycin in combination with electro-pulse treatment. A high dose, one-tenth of the LD50, did not improve the treatment outcome, probably due to a suppressed immune system caused by the high Bleomycin dose.[109]

Table 5-1
Summary of treatment protocols and outcome for electro-pulse-enhanced chemotherapy performed on liver tumours.

Tumour (animal)	Volume at treatment mm^3	Pulse parameters E V/cm	t (ms)	N
Hepatoma (rat)	220	1000	0.1	6
Colon (rat)	24	1300	0.1	6
Papilloma (rabbit)	380	850	0.1	?
Colon (mouse)	visible	1000	0.1	8
Colon (rat)	300	1300	1.0 Exp.	8+8

Table 5-1 (continued)

Study	Dose (BLM) mg/kg	Response Cure rate	CR rate
Jaroszeski, 1997[36]	1.7 (i.t)	ND	69%
Chazal, 1998[37] [34]	2.25 (i.t)	22 %	
Ramirez, 1998[110].		30 %	
Kuriyama, 1999[109]	0.5-1.0 (i.v.)	0 %	
Engstrom, 2001.[76]	23 (i.t)	92 %	

The study by Engström *et al*. (2001) reports better treatment results than the others.[76] The protocol used differs from the others in terms of the pulse duration and the number of pulses. Exponential pulses of 1.0ms time constant were applied, which have shown to result in higher drug uptake in tissue compared to 0.1ms square pulses.[90] The square wave pulses are less damaging to the healthy tissue surrounding the tumour, but may not always be as effective in tumour treatment. No increase in the alanine-aminotransferase level appeared, and an overall gain in weight supports the view that the treatment trauma was mild. At 40 days after treatment, the tumours were almost wholly eradicated with only minor fibrous tissue remnants.

5.2 PRECLINICAL STUDIES OF CISPLATIN CHEMOTHERAPY

A study of cell survival in vitro was performed to determine whether electroporation may increase the absorption of various anticancer drugs, and thereby increase their toxicity to human colorectal cancer cells.[111] The 50% survival level of cancer cells decreased by 100-1000 times after treatment with Bleomycin and electroporation, but only by 2-5 times when using 5-fluorouracil, and not at all with Cisplatin. Cancer cells transplanted subcutaneously onto nude mice and treated with a combined treatment of Bleomycin and electroporation, caused, after 3 weeks, reduced tumour size[111] Another study was performed to determine whether electroporation may increase the absorption of various anticancer drugs and thereby increase their toxicity to gastrointestinal cancer cells. The 50% survival level of the cancer cells decreased by a factor of 10000 after treatment with

Bleomycin and electroporation, but only with a factor of 10 when using Cisplatin or 5-fluorouracil. Cancer cells transplanted subcutaneously onto nude mice and treated with a combined treatment of Bleomycin and electroporation, caused, after 3 weeks, reduced tumour size.[112]

In vitro electroporation treatment increases the cytotoxicity of Cisplatin in human ovarian cancer and also in its resistant sub-clone.[113] In another study of mice treated with electroporation in 3 groups, only intra-tumoural (i.t.) administration of Cisplatin, and a combination of electroporation and i.t. Cisplatin, resulted in any improvement. Treatment performed with only i.t. Cisplatin resulted in up to 20 days' delay in tumour growth, but a combined treatment of electroporation and i.t. Cisplatin resulted in two times more tumour control.[114]

In addition to Cisplatin (CDDP), there is another platinum-based chemotherapeutic: Oxaliplatin (OXA). A study comparing the effect of these compounds in combination with electroporation showed that CDDP was nearly twice as effective as OXA. The study also showed that electro-pulse-enhanced chemotherapy with platinum-based chemotherapeutics increases lymphocyte infiltration in the tumour.[115]

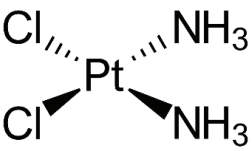

Figure 5-1
Cisplatin (CDDP)

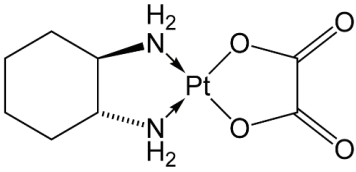

Figure 5-2
Oxaliplatin (OXA)

5.3 *EpEChT* AND CYCLOPHOSPHAMIDE (CTX)?

Cyclophosphamide has been found to induce beneficial immunomodulatory effects on adaptive immunotherapy. The suggested mechanisms involved in the creation of this immunologic effect were the following events:[116]

- Elimination of T regulatory cells (CD4+, and CD25+ T cells) in naive and tumour-bearing hosts;
- Induction of T cell growth factors, such as type I interferons (IFNs);
- Enhanced grafting of acquired transferred, tumour-killing effector T cells (CTL).

Thus, cyclophosphamide preconditioning of recipient hosts (for donor T cells) might be used to enhance immunity in naïve hosts.

Since cyclophosphamide has been found to induce beneficial immunomodulatory effects in immunotherapy, electro-enhanced chemotherapy using cyclophosphamide in combination with Bleomycin would be an effective tumour treatment modality as well. A recent in vitro study showed that electro-pulse-enhanced chemotherapy using cyclophosphamide (CTX) has a similar anti-tumoural effect to Bleomycin.[117]

The combination of electro-enhanced gene-therapy with Vasostatin-gene and cyclophosphamide administration was found to inhibit tumour growth, and resulted in extended survival in mice with melanoma tumours.[118]

5.4 ELECTRO-PULSE ENHANCED IMMUNO THERAPY

5.4.1 IMMUNE RESPONSE OF *EpEChT*

Immunogenic Fibrosarcoma treated with electro-pulse enhanced chemotherapy using Bleomycin in immunocompetent syngeneic mice and immune-deficient (nude) mice, resulted in only a temporary delay in tumour growth.[103] Further studies showed that the host's immune system activates after tumour treatment with electro-permeabilization.[119] Mir and colleagues achieved a systemic, anti-metastatic immune response by delivering histo-incompatible cells secreting interleukin-2 (IL-2) in combination with electro-pulse enhanced chemotherapy to 3LL-Lewis lung carcinoma in mice.[120] In another study, a stimulated immune response, through increased monocyte and T-lymphocyte activity, was found by using Cisplatin instead of Bleomycin.[121]

Genetically engineered IL-2-secreting cells injected intratumourally or in peri-tumoural oedema after electro pulse enhanced chemotherapy of VX2 tumours transplanted into rabbit liver increased the cure rate from 30% to 40%. More importantly, treatment resulted in a clear anti-metastatic effect on tumour spread in the liver and lungs, which led to cures also of the untreated contralaterally implanted tumours. This systemic antitumor effect also resulted in immunity with complete protection against further tumour cell inoculation.[122]

The results of treatment of liver tumours also imply that electro-pulse enhanced chemotherapy stimulates a response in the host’s immune system against the tumour.[76] Two weeks after electro-pulse-enhanced chemotherapy of colorectal tumours in the liver, with exponential decaying pulses of 1.0ms time constant and Bleomycin, macrophages, T-lymphocytes were found in

excised stained viable tumours.[76] The results showed overall low levels of CD4 positive lymphocytes and ED2 positive macrophages. The most significant amounts of CD8 positive lymphocytes were found in a viable tumour treated with electro-pulse enhanced chemotherapy with Bleomycin, and the lowest amounts in tumours treated with Bleomycin only. The most substantial amounts of ED1 positive macrophages were found in tumours treated with electrical pulses only and combined with Bleomycin. The electro-pulse chemotherapy treatment also seemed to have anti-metastatic effects. Intra-abdominal metastases developed, however, after surgical excision of the tumour-bearing lobe. At 14 days after electro-pulse enhanced chemotherapy treatment, none out of 13 treated animals showed signs of tumour spread. These results strongly suggest that electro-pulse-enhanced chemotherapy stimulates activation of the host's immune system against a tumour and prevents metastases development.[76]

Calvet and collaborators showed in 2014 that the injection of CT26 murine colon cancer cells, in vitro treated with *EpEChT*, elicits an antitumor immune response that prevents the growth of a subsequent administration of viable cancer cells. The treatment caused complete regressions in immunocompetent animals but not in immune-deficient ones, which supports the role of immunity in allowing for a beneficial outcome of electro-pulse-enhanced cancer therapy.[123,124]

5.4.2 ELECTRO-PULSE THERAPY WITH IL-18 AND IFNγ SECRETING CELLS

The brain tumour models used in electro-pulse-enhanced immunotherapy treatment are two strains of rat brain glioma: N32 and N29 were chemically induced (ethyl-N-nitrosourea) and developed at the Department of Tumour Immunology of Lund University, Sweden. These tumours are only weakly immunogenic and grew intra-cerebrally, subcutaneously and in vitro.[125] In the present study, the tumours were created by subcutaneously injecting 100 000 tumour cells into the thigh of the rats' hind legs. After 4 weeks, a solid tumour developed with a diameter of 1 to 1.5 cm, and the animals were then treated.

In an attempt to achieve immunoreactions against implanted brain tumours, rats with N29 glioma tumours were treated with electric pulses followed by injections of IL-18 and IFN-γ secreting cells.[126]

Tumours were inoculated subcutaneously on both thighs of female Fischer-344 syngeneic rats. The left tumour was treated once with 16 pulses of 1400 V/cm with durations of 1.0ms (time constant), without anticancer drugs given at any time. The following day, and then once a week for three

weeks, the animals were given intraperitoneal injections of irradiated, modified N29 tumour cells, secreting either interleukin-18 (IL-18) or interferon-gamma (IFN-γ).

No effect in contralateral tumour growth appeared in animals given no treatment, or treatment with only electric pulses, with only IFN-γ secreting cells and with only IL-18 secreting cells. Having said this, an inhibited growth rate of contralateral tumours appeared in animals given electric pulse treatment followed by intraperitoneal injections of IL-18 secreting cells. This treatment resulted in prolonged survival (the time for a contralateral tumour to reach the predetermined limit volume) of 50%.

These results show that the systemic response of the host's immune system can be achieved against a tumour using *syngeneic* tumour cells. This is an essential step towards the development of electro-pulse-enhanced immunotherapy for an effective tumour treatment modality.[126]

5.4.3 EpEIT WITH DAC-TREATED IL-18 AND IFNγ SECRETING CELLS

The DNA de-methylating agent 5-aza-2-deoxycytidine (DAC) is a cytosine analogue that covalently binds to DNA methyl-transferase and inhibits its activity in causing de-methylation. The gene expressions which may be silenced by the hypermethylation of their promoters are re-activated by DAC. Expressions of HLA class I are significantly increased in DAC-treated glioma cells when compared to those of untreated cells, which makes DAC a potent immune stimulator.

These findings provide the basis to consider the use of 5-aza-2-deoxycytidine (DAC) treatment of IFN-γ transfected tumour cells aiming for immune therapy. The study was performed in Fischer 344 rats with N29 glioma tumours subcutaneously implanted on both flanks to study the immunoreactions against implanted brain tumours. After treatment of a contralateral tumour with pulsed electric fields (PEF), injections of IL-18 or IFN-γ secreting cells followed. The following day, and once a week for three weeks, the animals received intraperitoneal injections of radiation sterilized, gene-modified N29 tumour cells, secreting either interleukin-18 (IL-18) or interferon- (IFN-γ).

In one group of animals, IFN-γ transfected cells treated with DAC were used for immunization. The size of the tumours on the flank was measured daily to evaluate the tumour growth-rate (TGR) regarding the specific therapeutic effect (STE), which is the difference in tumour growth-rate between the control and exposed tumours, divided by the tumour growth rate of the controls. The therapeutic enhancement ratio (TER) expresses the

quantitative estimate of the sensitisation effect of the combination of pulsed electric fields and radiation therapy. TER is the ratio of the STE of the experimental combination of pulsed electric fields (PEF), and immunization and STE of the hypothetical independent combination of the two agents.[127]

Treatment with only PEF in 32 animals resulted in a specific therapeutic effect of 20±6 %. The effect on the contralateral non-targeted tumour was negligible (0±4 %).

Immunization with IFNγ secreting cells in 27 animals showed a slightly increased growth-rate of both the left and right tumours. This adverse effect is probably due to strong immune-suppression caused by the heavy tumour burden. However, immunization with DAC treated IFNγ secreting cells (IFNξ) in 12 animals showed decreased growth-rates on both the left (10%) and right (5%) tumours. Thus, the DAC treatment had induced such a strong antigen expression that the immune response was partly able to overcome the immune-suppression.

For the combination of PEF+IFNγ, there was no therapeutic enhancement, but in the combination of PEF and IFNγ secreting cells grown in a 5-Azacytidine medium (IFNζ), the therapeutic enhancement factor was about 2 on both sides. However, for the combination of PEF + immunization with DAC-treated IFNγ secreting cells (IFNξ), there was a sizeable therapeutic enhancement in the combination of PEF and IFNξ (50%), and on a non-targeted tumour there was an effect of about 20%.

Thus for the combination of PEF + IFNγ, there was no therapeutic enhancement, but in the combination of PEF and IFNγ secreting cells grown in a 5-Azacytidine medium (IFNζ), the therapeutic enhancement factor was about 2 on both sides of the tumours.

5.5 ELECTRO-PULSE ENHANCED RADIATION THERAPY "*EpERT*"

5.5.1 *EpERT* TREATMENT OF FIBROBLAST CELLS

The remarkable effective killing of V-79 fibroblast cells appeared after combined exposure with 2Gy radiation (RAD) and exponentially shaped, pulsed electric fields with an amplitude of 1600V/cm and a time constant of τ=1ms at 1Hz repetition (PEF).[128,77,129,130]

Radiation therapy alone is known to have a limited therapeutic effect on brain tumours, but the combination of ionizing radiation exposure with high voltage pulses was never studied before. Encouraged by the results from V-79, we carried out the combined treatment PEF+RAD on a rat brain tumour

model. In the study of tumours created by subcutaneously injecting 100 000 N29 brain tumour cells onto the thigh of the rat´s hind leg, after 4 weeks, a solid tumour developed with a diameter of one to 1.5 cm. The animals were then treated.

The Fischer rats with N32 tumours grown on their flanks received four daily combined treatments with RAD+PEF and the reversed PEF+RAD. The tumour response was evaluated by observing the change of tumour volume measured as an ellipsoid (as is shown in Figures 5-3 and 5-4).

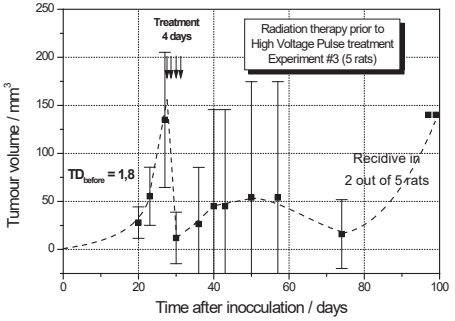

Figure 5-3
Average effect in five rats of combined treatment first with ^{60}Co-radiation 5 Gy and then with 16 high voltage pulses (1300V/cm. τ=1ms), over 4 consecutive days

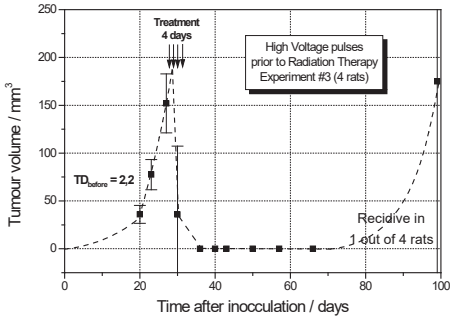

Figure 5-4
Average effect in four rats of combined treatment first with 16 high voltage pulses (1300 V/cm. τ=1ms) and then with ^{60}Co-gamma radiation 5Gy, over 4 progressive days

5.5.2 *EpERT* OF SUBCUTANEOUS IMPLANTED N29 TUMOURS IN FISCHER RATS

Another experiment arranged Fischer rats with subcutaneous implanted N29 tumours into five groups. One group of 8 rats were controls (Ctrl) and received no treatment. A treatment group (RAD) with 5 rats received radiation (4×6 Gy), and a treatment group PEF with 4 rats received pulsed electric fields (4×8 pulses of 1400 V/cm). The groups of combined treatment (PEF+RAD) with 9 rats received pulsed electric treatment combined with a total absorbed radiation dose of 20Gy, given in four daily fractions of 5Gy each.

Animals (single-treated and controls) put to death when a tumour had reached a size of 5 cm^3. Table 5-2 shows the averages of the time until this event as "survival time" for the various groups. There was a significant difference ($p<0.05$) in survival time between the combined treatment and the control groups. Fisher's exact probability test of 2×2 contingency tables was used to calculate p-values of significant differences. The number of cures was significantly larger for the EP+RT group compared to the control group ($p=0.009$) and the RAD group ($p=0.03$). There were no significant differences in the number of cures for PEF as opposed to controls or PEF vs. RAD.

Results from combined treatment with ^{60}Co-gamma radiation therapy (RAD) and pulsed electric field therapy (PEF) show the average time when a tumour had reached a size of 5cm^3:

> 94 days when treated first with RAD then PEF
>100 days when treated first with PEF then RAD

Table 5-2
Treatment data, a summary of results and statistics

Treatment	*Survival (days) excl. cures*	TGD (%) *of non-cured*	*N*	*Number of cures (CR>80 d Post treatm.*	*Fisher's exact probability test*
Control	41.5 ± 1.1	0	8	0	
RAD	50 ± 1.1	21	5	0	NS (vs.Ctrl.)
PEF	70 ± 14.6	69	4	1	NS (vsCtrl.)
PEF+RAD	98.3 ± 0.7	137	9	6	P<0.01 (vs.Ctrl.) p<0.05 (vs.RAD)

All treated animals showed an initial partial or complete tumour remission within a few days after the end of the 4-day treatment for the combination radiation RAD (4×5Gy) and then PEF (4×1300V/cm). Three out of the 11 treated animals had complete remission lasting more than 100 days. For the combination (4×PEF1300V/cm) and then radiation (4×5Gy), 3 out of the 4 treated animals had complete remission >100 days. The outcome of this preliminary study of combining pulsed electric field and radiation therapy suggests an astonishingly effective therapeutic response.[131,77,132,80]

5.5.3 T-LYMPHOCYTE INFILTRATION IN TUMOURS

Infiltration of T-cells CD4+ and CD8+ was studied in subcutaneous implanted N29 tumours after various types of treatments.[78,90] The tumours were excised after treatment and embedded in paraffin. The scanned images shown in Figure 5-5 and 5-6 below are microscopic slides scanned in a CCD scanner, and the degree of staining evaluated by Image processing (Image Pro 5).

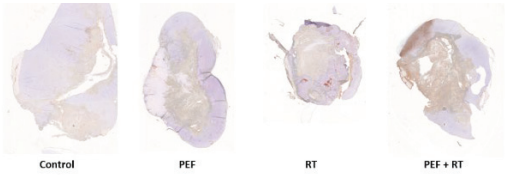

Figure 5-5 CD4+ infiltration in tumours of various treatment: Controls, pulsed electric fields PEF (1400 V/cm), radiation therapy RT (4x5 Gy) and the combination PEF+RT (*EpERT*)

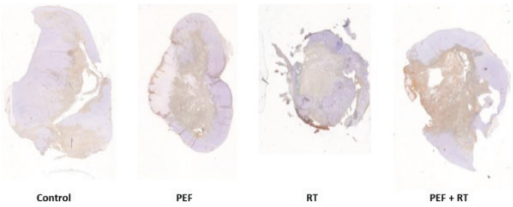

Figure 5-6 CD8+ infiltration in tumours of various treatment: Controls, pulsed electric fields PEF (1400 V/cm), radiation therapy RT (4x5 Gy) and the combination PEF+RT(*EpERT)*

Figures 5-7 and 5-8 show the average results of the area of a tumour stained for CD4+ CD8+ respectively after a number of different treatments for each group.

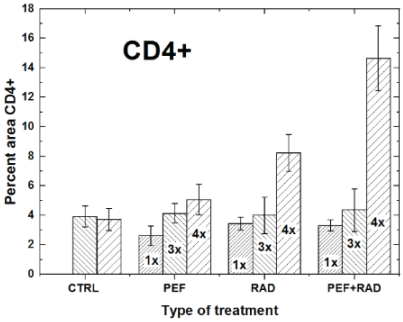

Figure 5-7
Percent area of tumours stained for CD4+ after a number of different treatments: Controls (CTRL), pulsed electric fields (PEF 1, 2, and 4x 8 pulses of 1400 V/cm), radiation therapy (RAD 1, 3 and 4x5 Gy), and a combination (PEF+RAD 1, 3, and 4 times of each).

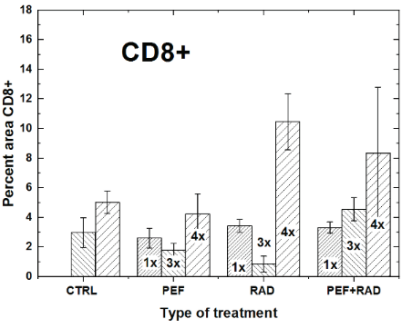

Figure 5-8
Percent area of tumours stained for CD4+ after a number of different treatments. Controls, pulsed electric fields PEF (1, 2, and 4x 8 pulses of 1400 V/cm), radiation therapy RT (1, 3, and 4x5 Gy) and the combination PEF+RT,

By using the presented system for treating tumoural diseases (cancer) with a combination of ionizing radiation and pulsed electric fields, a new regime of radiation fractionation is possible. A short intensive treatment using high radiation dose fractions combined with pulsed electric fields followed by a longer resting interval allows the patient's own immune system to be effectual. The intensity of the following therapy session may depend on the outcome of the previous treatments. Recurrent tumours already given full dose with conventional therapy could receive further radiation fractions up to a total absorbed dose of less than 15Gy combined with pulsed electric fields. It is thus worthwhile to further explore this combined type of treatment to improve the therapy of resistant tumours. It might be of particular interest to apply high voltage impulses combined with brachytherapy where the radioactive source (i.e., Iridium-192) can serve as an electrode.[133]

5.6 *EpEChT* COMBINED WITH RADIATION THERAPY

5.6.1 EAT TUMOURS IN MICE WITH CISPLATIN AND RT

Sersa and colleagues studied the combined modality of electro-pulse enhanced chemotherapy with Cisplatin and radiation using subcutaneous

Ehrlich ascites tumours (EATs) in CBA mice.[134,135] The mice were treated either with Cisplatin (CDDP), electric pulses (EP), or ionizing radiation (RT), as well as combinations thereof. Table 5-3 shows the results of the combined treatments recorded 100 days after treatment. These results show that delivery of Cisplatin into the cells through electroporation of tumours increases the radio-sensitizing effect of Cisplatin. However, the effect may also be due to the radiation reduced immunosuppression through a tumour which enhances the effect of electro-pulse chemotherapy.

Table 5-3
The published results by Sersa et al. (2000).[135]

Treatment	Complete remissions / %
Control	0
CDDP 4 mg/kgBW	0
EP (8x1300 V/cm. 0.1ms)*	0
EP+CDDP	12
RT (15 Gy)^	37
EP*+RT^	54
RT^+CDDP	73
EP*+CDDP+RT^	92

5.6.2 TREATMENT OF LPB SARCOMA IN MICE WITH CISPLATIN *EpEChT* and RT

In a study in 2003, Kranjc and colleagues treated LPB murine sarcoma cells and tumours in mice either with Cisplatin, electroporation or ionizing radiation, as well as combinations of these. In vitro response was determined by colony forming assay, while in vivo treatment effectiveness was determined by the values of the 50% tumour control dose (TCD_{50}).

They found that exposure of cells in vitro to a combination of Cisplatin and electroporation increased and prolonged accumulation of Cisplatin and reduced tumour perfusion. Combined with ionizing radiation, the effectiveness of the treatment is significantly enhanced by 60%, compared to the irradiated tumours, and 40% compared to tumours treated with Cisplatin and irradiation.[136]

5.6.3 TREATMENT OF LPB SARCOMA IN MICE WITH *EpEChT* BLEOMYCIN, and RT

In a study in 1995, Okunieff et al. investigated the effect of Bleomycin *EpEChT* combined with radiation therapy (RT) on subcutaneous Murine

sarcoma LPB tumours in C57Bl/6 mice.[137] Three minutes after intravenous injection of BLM (injection volume 150µl), 8 electric pulses of 1300 V/cm with 0.1ms duration at 1 Hz were applied to the tumours using plate electrodes placed on the skin overlying the tumour.

Table 5-4
The values of the 50% tumour control dose (TCD50) and the relative tumour control (RTC) versus radiotherapy RT only for various combinations of treatment.[137]

Treatment	TCD_{50}	SD	RTC
RT	23.1	0.3	1.0
EpE+RT	22.1	0.3	1.0
Bleo+RT	22.8	0.3	1.0
*EpE*Bleo+RT	12.4	0.3	1.9

They used the TCD_{50} value (i.e., the dose that decreases the treated tumour to half its size) as a measure of the therapeutic effect.[137] Table 5-4 shows the values of the 50% tumour control dose (TCD_{50}) for various combinations of treatment and the relative tumour control (RTC) versus radiotherapy RT only.

Neither treatment of animals with radiation alone (RT) nor the application of electric pulses or Bleomycin to the tumours before irradiation (*EpE*+RT and Bleo+RT) of tumours had any effect on the 50% TCD value (TCD_{50}) (Table 5-4). However, the application of electric pulses with Bleomycin combined with radiation therapy (*EpE*Bleo+RT) decreased the TCD50 value of tumours by a factor of 1.9 compared to any other treatment. Hence it is evident that radiation therapy of tumours significantly contributed to the therapeutic effect of Bleomycin *EpE*CT.[138]

In a 2009 study, Kranjc et al. evaluated the interaction between electrochemotherapy using Bleomycin and single-dose or fractionated radiation in two murine tumour models with different histology.[139] They found that electro-pulse chemotherapy before radiotherapy of the tumours potentiated the response to a single fraction ionizing radiation of 10Gy or five daily fractions of 2Gy.

Figure 5-9 shows the results of the data for the radiosensitive sarcoma SA-1 extracted from *Figure 2A* of Kranjc's 2009 publication and normalized to 100% at the beginning of treatment fitted to Boltzmann´s logistic functions.[138]

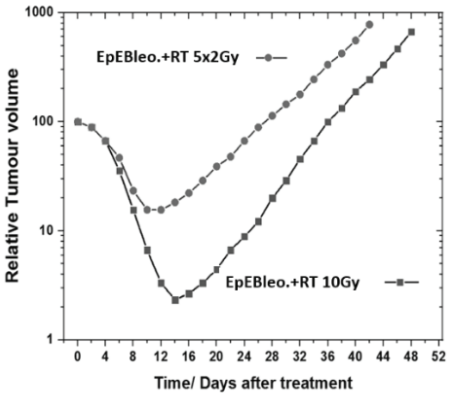

Figure 5-9
The tumour growth curves after treatment with Bleomycin *EpEChT* combined with radiation therapy in a single fraction of 10Gy or five daily fractions of 2Gy.[138]

Figure 5-10a shows the decrease of tumour volume during the first 15 days after treatment with Bleomycin *EpEChT*, and a single fraction radiation RT10Gy, and Figure 5-11a offers the corresponding curve after *EpEChT,* and five fractions of radiation RT5x2Gy.[139]

Figure 5-10b shows the increase of tumour volume during the regrowth period, starting at 15 days after Bleomycin *EpEChT*, and a single fraction radiation and Figure 5-11b shows corresponding curve after *EpEChT* and five fractions of radiation +RT5x2Gy.[139]

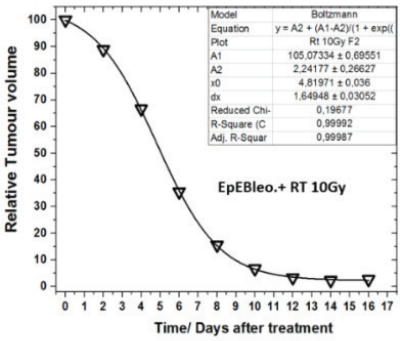

Figure 5-10a
The decrease of tumour volume in the first 15 days after treatment with Bleomycin *EpEChT*+RT10Gy, and the data fitted to the Boltzmann sigmoid equation.

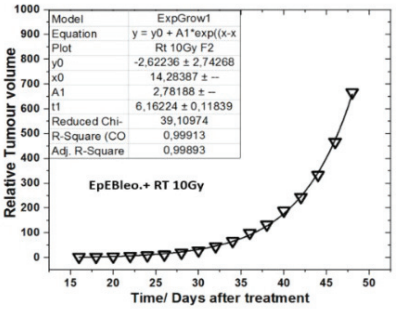

Figure 5-10b
The increase of tumour volume during the regrowth from 15 days after Bleomycin *EpEChT*+RT10Gy treatment, and the data fitted to an exponential growth equation.

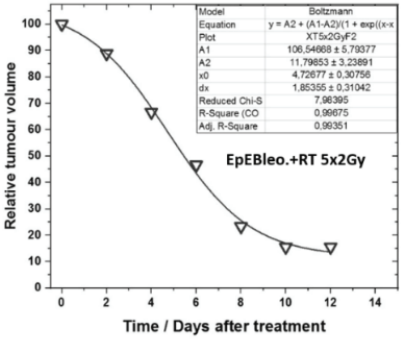

Figure 5-11a
The decrease of tumour volume in the first 15 days after treatment with Bleomycin *EpEChT* + RT5x2Gy, and the data fitted to the Boltzmann sigmoid equation

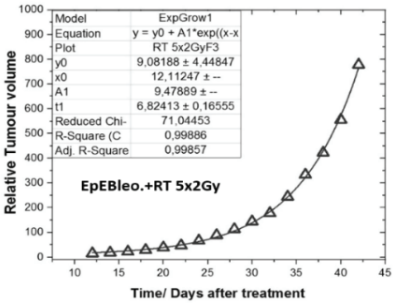

Figure 5-11b
The increase of tumour volume during the regrowth from 15 days after Bleomycin *EpEChT*+RT5x2Gy treatment, and the data fitted to an exponential growth equation

The minimum tumour volume after 14 days is about 2% for single fraction radiotherapy and about 15% after 12 days for fractionated radiation therapy. In both cases, however, there is a tumour regrowth with about 15% per day.

The mechanism behind the effect of the combination of *EpECT* ad RT is probably similar to the enhancement of the effect of tumour vaccine by a single fraction of RT.[140,141,142,143] The creation of a bolus of dead tumour cells as a result of the *EpEChT* treatment is similar to the administration of a bolus of dead tumour cells, which activates the tumour suppression through an accumulation of myeloid-derived suppressor cells (MDSC) in the tumour microenvironment. A single fraction of ionizing radiation of about 8 Gy deactivates the immune-suppressive effect during the following 10-14 days. If there are surviving tumour cells present after the treatment, the tumour starts growing again. Thus, several repeated treatment regimens are required to achieve a complete cure.

The results of the combination of *EpEChT* and radiation therapy suggest a therapy regime with repeated treatments of EpEChT+RT once a week until a complete cure appears. The absorbed dose and number of treatment procedures required might vary with the histology of a tumour and from patient to patient. Thus, the oncology community should urgently start clinical trials to standardize the treatment parameters for the combination of electro-pulse enhanced chemotherapy and radiation therapy.

5.7 PRECLINICAL STUDIES OF ELECTRO-ENHANCED THERAPEUTIC EFFICIENCY OF OTHER DRUGS

5.7.1 ELECTRO-ENHANCED THERAPEUTIC EFFECTS OF CALCIUM

Calcium is essential for plant cells, as well as for mammalian cells. However, an overload of intracellular calcium-ions is toxic already at small concentrations deviating from normal conditions.[144,145]

In 1995, weed control experiments were performed with seeds of *Sinapsus Alba* as a model to investigate the effect of high voltage pulses at various calcium concentrations.[146,147]

The seeds of Sinapsis Alba were first steeped in ordinary tap water during 40 minutes and then placed in solutions of different calcium concentrations (0.05, 3, 10 and 50mM). After treatment with pulsed electric fields of various field-strengths ranging from 500 to 3000 V/cm, the seeds were set to grow in a climate controlled environment to examine their survival. A control group was established with seeds treated in the same way as above, but without electric field exposure. After treatment, the seeds were placed on wet filter paper in petri-dishes, and set to grow during 7 days for evaluation with the sprout length (SL) as a measure of survival.

Figure 5-12 shows the survival fraction (SF = SL(EP-exposed)/SL(control)) for seeds of ***Sinapsis Alba*** steeped with various Ca-concentrations and then treated with 20 exponential pulses (1ms time-constant) at various electrical field-strengths. Figure 5-13 displays the survival fraction for seeds of *Sinapsis Alba,* steeped with various concentrations of calcium ions and then treated with 20 exponential pulses (1ms time-constant) at 1000V/cm.

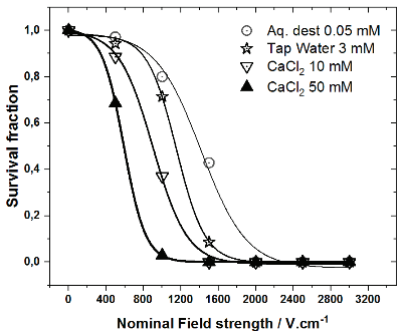

Figure 5-12
Survival curves for seeds of *Sinapsis Alba* steeped with various Ca-concentrations and then treated with 20 exponential pulses (1ms time-constant) at various electrical field-strengths.

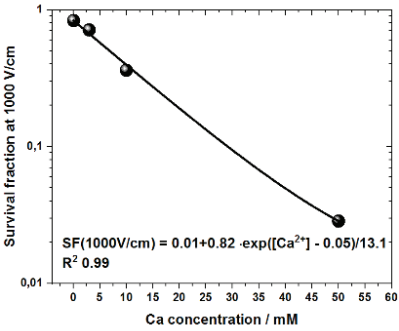

Figure 5-13
Survival fraction for seeds of *Sinapsis Alba* steeped with various Ca-concentrations and then treated with 20 exponential pulses (1ms time-constant) at 1000V/cm.

These results clearly show the enhanced seed killing effect of increased Ca-concentration in the electro pulse medium. The effect is believed to depend on a decreased threshold membrane potential for dielectric breakdown. A study exploring how electric field pulses can induce apoptosis recorded that divalent ions in the medium apoptosis occurred at much lower (about 10 times) concentrations than monovalent ions.[148]

In 2014, Frandsen et al. confirmed the Ca-effect in three different cell lines (DC-3F), and transformed Chinese hamster lung fibroblast; K-562, human leukaemia; and murine Lewis Lung Carcinoma. With the aim to apply this finding in electro-enhanced cancer therapy they clinically investigated the effects of electrical pulsing parameters and calcium as further reviewed in the next chapter.[149,150,151]

5.7.2 ELECTRO ENHANCED THERAPEUTIC EFFICIENCY OF MITOMYCIN C

Human pancreatic tumours (Pan-4-JCK) were implanted onto nude mice treated with Bleomycin, Mitomycin C or Carboplatin as single agents in electro-pulse chemotherapy. The therapeutic effect on tumour growth monitored for 89 days was in the order Bleomycin >> Mitomycin C > Carboplatin.[152]

Mitomycin C electro-pulse treatment of a T24 bladder cancer cell line enhanced the cytotoxicity by about 30%, which supports the use of this combination in the treatment of bladder cancer.[153] Further in vivo studies revealed a tumour response rate of 100% for electro-pulse treatment in combination with Mitomycin C with significant tumour volume reduction.[154]

CHAPTER VI

CLINICAL STUDIES OF ELECTRO-PULSE-ENHANCED CHEMOTHERAPY

6.1.1 INTRODUCTION

In the early 1990th Belehradek and collaborators performed an initial clinical trial with Bleomycin, followed by 4 or 8 electric pulses of 1.3 kV per cm electrode distance, and 0.1ms in duration, resulted in an apparent antitumor effect, reaching 72% of objective responses OR, with 57% of complete response CR.[155,94] The first Phase I-II clinical trial of electro-chemo-therapy performed in 8 patients with 40 nodules of head and neck squamous cell carcinomas, previously irradiated and surgically treated without cure and with tumours resistant to conventional chemotherapy. The patients were given Electro-pulse enhanced chemotherapy with an intravenous bolus of 10 mg/m^{2}BSA.[156]

Another clinical study was performed in a group of 34 patients, to determine whether the dose of intra-lesional administered Bleomycin based on tumour volume in combination with electric pulses could improve the treatment result. The Figures 6-1a and 6-1b display the dose of Bleomycin versus tumour volume.[32]

In Figure 6-1 the dotted line is the graph of the fitted "*Dose response equation*" (Eq. 6.1).

$$\text{Dose} = A1 + (A2-A1)/[1 + 10^{(LOGx0-TV)\cdot p)}] \qquad \text{Eq.6.1}$$

R-Square(COD)=0.98

where:

A1 = bottom asymptote =-4123;,
A2 = top asymptote =4147,
LOGx0 = centre =-574,
TV = Tumour Volume,
P = hill slope =$2.15 \cdot 10^{-4}$,

Figure 6-2 displays the Bleomycin data in USP units fitted to a 2nd degree polynomial of the logarithm of tumour volumes (TV) between 100 - 5000 mm^3 Eq. 6.2.

$$\text{Bleo (USP units)} = 5.55 - 4.74 \cdot \log_{10}(TV) + 1.147 \cdot (\log_{10}(TV))^2; \quad \text{Eq.6.2}$$

R-Square(COD) =0.98.

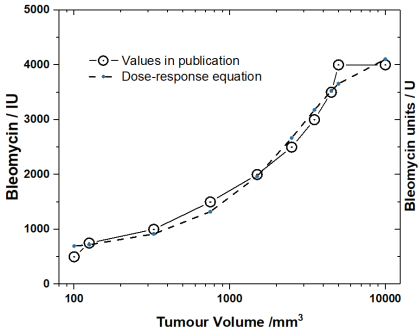

Figure 6-1
The open circles are data extracted from the publication[32], and the dashed line is the graph of the fitted equation..

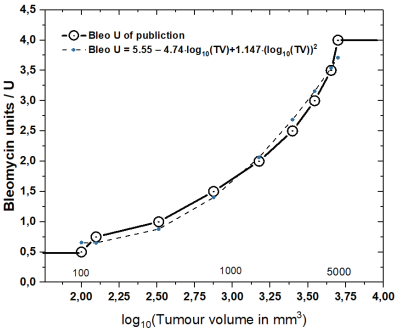

Figure 6-2
The open circles are data extracted from the publication[32], and the dotted line is the graph of the fitted equation.

At 10 minutes after the Bleomycin administration six to eight electric pulses of 1.3 kV amplitude per cm electrode distance and 0.1 ms in duration delivered, and within 12 weeks complete responses observed in 91% of the treated nodules.[32]

From January 1995 to August 1996, a clinical study with electro-enhanced chemotherapy enrolled 29 male and five female patients (mean age, 62.2 years) with BCC, advanced melanoma metastatic to local or distant soft tissue and skin sites, squamous-cell carcinoma (SCC), or Kaposi's sarcoma.[157]

1998, Mir et al. summarized the results of independent clinical trials, performed by five cancer.[31] In those trials, a total of 291 cutaneous or subcutaneous tumours of basal cell carcinoma (32), malignant melanoma (142), adenocarcinoma (30) and head and neck squamous cell carcinoma (87) treated in 50 patients. Complete response appeared in 56% of the treated tumours, and partial response in 29% of all tumours treated.

A phase II prospective clinical trial took place during 1998-1999, that involved two patients with nine nodules of basal cell carcinomas, two patients with 13 metastases of melanoma, two patients with two nodules of

squamous-cell carcinomas (SCC), and two patients with 14 skin metastases from breast cancer. Intra-lesional Bleomycin administered according to Figure 6-1, and 10min after Bleomycin injection, electric pulses at a field strength of 1300V/cm and pulse-length of 100μs were delivered at a frequency of 1Hz. The mean follow-up was 8.6 months, with overall objective responses of 98%, complete or partial responses of 49%, and stable disease (i.e., no response) in 2%. In the two patients with basal cell carcinoma, a 78 % complete response was achieved.[158]

A review of electro-enhanced chemotherapy with Bleomycin in clinical studies involving 96 patients with altogether 411 malignant tumours reported a wide spread of the fraction of complete remissions between 9-100 % depending on the technique used in the treatment. The results indicate that the treatment, for the most part, is palliative, while curative in the patients with basal cell carcinoma and solitary tumours without metastases.[159]

From the 1st of January 2003 to the 31st of December 2005 a European project "*European Standard Operating Procedures of Electro-chemotherapy*" (ESOPE), was launched with the aim of prepare standard operating procedures (SOP) for electro-enhanced chemotherapy. The project based on the electro-pulse enhanced chemotherapy activities of four European cancer clinics.

The final ESOPE recommendations are summarized in a number of publications in *European Journal of Cance*r, EJC Supplements 4 (2006).[160,161,162,163,164,165,166,167,168]

An updated standard operating procedure for electrochemotherapy of cutaneous tumours and skin metastases was recently released.[169] This updated ESOPE considers recommendations for indications and treatment choices for electro-enhanced chemotherapy, and information about pre-treatment, evaluation, and patient follow-up.[169]

Anaesthesia

Consider local anaesthesia if tumour count ≤ 7 and size ≤ 3cm
Consider general anaesthesia if tumour count > 7 and size > 3cm.[169]

An intravenous administration

ESOPE recommends intravenous administration of Bleomycin to a dose of 15000 IU per m^2 body surface, 15 000 IU (International units) = 15 USP units = 15 mg.

An intra-tumoural administration

Bleomycin at a concentration of 1000 IU/ml is recommended by ESOPE to a dose depending on tumour volume according to the following rules:[165 ,169]The injection volume depends on the size of the tumour nodules = TV cm^3,

Injection volume (ml) = Bleo i.t. dose (IU) / 1000 (IU/ml)

For tumour > 1 cm^3 250 IU/cm^3, Bleo i.t. Dose = 250 ×TV [IU]

For tumour 1 – 0.5 cm^3 500 IU/cm^3, Bleo i.t. Dose = 500 ×TV [IU]

For tumour < 0.5 cm^3 1000 IU/cm^3. Bleo i.t. Dose = 1000 ×TV [IU]

Parameters for electric pulse treatment with the type of electrodes:

Electric pulses should be applied to the tumour nodules in a time window between 8 and 28 min after the intravenous injection of Bleomycin, or as soon as possible within 2 min after its intra-tumoural injection.

Plate electrodes.

8 electric pulses of 1.3 kV amplitude per cm electrode distance and 0.1ms in duration, delivered at either 1Hz or 5 kHz repetition frequency;

Four needle electrodes placed in parallel rows.

8 electric pulses of 1 kV amplitude per cm electrode row distance and 0.1ms in duration, delivered at either 1Hz or 5 kHz repetition frequency;

Seven needle electrodes which are hexagonally centred.

96 electric pulses (eight pulses per pair of needles) of 1 kV amplitude per cm electrode distance and 0.1 ms in duration, delivered at either 1Hz or 5 kHz repetition frequency.[162]

To further improve the treatment results of electro-enhanced chemotherapy particularly of larger tumours, the present author suggests a new dimension of intra-tumour administration of Bleomycin:

Instead of a decreasing concentration with tumour volume as is recommended by ESOPE, the Bleomycin concentration could be the same for all tumoural volumes. The concentration of Bleomycin suggested is 500 IU per cm^3 of tumoural volume. A maximum dose limit of 40 000 IU that corresponds to a tumour volume of 80 cm^3, and 10% of the maximal allowed

i.v. Bleomycin dose, avoid too high doses. In order to avoid injection of too high volumes the concentration of the Bleomycin preparation for tumours larger than 5cm^3 could increase.

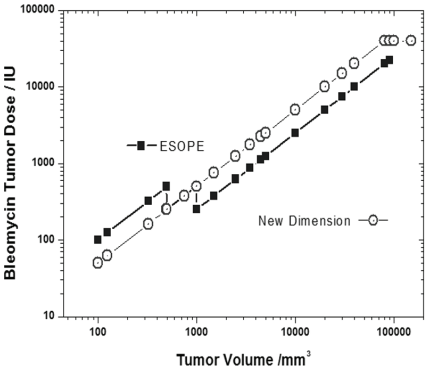

Figure 6-3
Bleomycin Dose (IU) in tumours of various volume (mm^3) at intra-tumoural administration according to the ESOPE recommendations,[165] and according to a constant Bleomycin concentration of 500 IU/cm^3 in a tumour up to a dose-limit of 40,000IU.

In recent years several authors have reviewed the clinical results of Electro Enhanced Chemotherapy:

- Electro-enhanced chemotherapy for primary skin cancer and skin metastasis related to other malignancies[170].
- Utility of electro- enhanced chemotherapy in melanoma treatment[171].
- Antitumor effectiveness of electro- enhanced chemotherapy: A systematic review and meta-analysis[172].
- Electro- enhanced chemotherapy in Breast Cancer: A review of references[173].
- Treatments of advanced basal cell carcinoma: a review of the literature"[174].
- Treatment efficacy with electro-enhanced chemotherapy: A multi-institutional prospective observational study on 376 patients with superficial tumours"[175].
- Basal cell carcinoma: 10-year experience with electro-pulse enhanced chemotherapy[176].
- Electro-pulse enhanced chemotherapy in Breast Cancer - Discussion of the method and literature review[177].

The treatment results are in most publications reported regarding the following quantities where the fractional response

$$f = (\text{No. cases with the actual response / Total No. Cases})$$

CR = Complete response;	f_{CR} = fractional complete response;
PR = partial response;	f_{PR} = fractional partial response;
SD = stable disease;	f_{SD} = fractional stable disease;
PD = progressive disease;	f_{PD} = fractional progressive disease;
OR = objective response;	f_{OR} = fractional objective response

Usually, the sum of complete and partial responses called an Objective Response (OR = CR + PR) is used as a general quantity for the response of the treatment in question without considering the responses of stable and progressive disease. This quantity, however, does not take into account the number of cases of progressive disease PR.

To create a quantity that estimate the response taking all four response quantities into account a quantity named "***fractional weighted response,"*** $\boldsymbol{f_{WR}}$, was previously defined as follows [178] :

$$WR_3 = (3 \cdot f_{CR} + 1 \cdot f_{PR} + 0 \cdot f_{SD} - 3 \cdot f_{PD})/3; \qquad \text{Eq. 6.3}$$

Here introduces a second attempt of defining a weighted response WR_4 which include all four response parameters, i.e., also the stable disease with weights according to the following equation 6.4 below.

$$WR_4 = (4 \cdot f_{CR} + 2 \cdot f_{PR} + 1 \cdot f_{SD} - 4 \cdot f_{PD})/4; \qquad \text{Eq. 6.4}$$

The following sections summarize the published responses of clinical electro-pulse enhanced chemotherapy for each specific type of a tumour and estimate the fractional weighting response WR_4 from the data extracted from each published report. The results are presented for each specific treatment device used for the treatment.

6.1.2. MALIGNANT MELANOMA

In 1996 Heller et al. initiated the first clinical study to determine if electro-pulse enhanced chemotherapy would be effective against specific primary and metastatic malignant melanoma.[179] The study enrolled a group of three patients with malignant melanoma. The objective response of electro-pulse enhanced chemotherapy (*EpEChT*) treatment of 11 tumours in the study was 47%, with 3 complete remissions (CR 25%), and 2 partial

remissions (PR 22%). The number of tumours with no response was 5 (42%).

Results were encouraging enough to continue the studies in another five patients with 35 metastases of malignant melanoma.[179] The objective responses of *EpEChT* treatment of these patients with intra-lesional Bleomycin-sulphate in 23 nodules were 95%, with 78% complete remissions (CR) and 17%, partial remissions (PR), while the fraction of *EpEChT* treated tumours with no response was 5%. In 9 nodules that received Bleomycin-sulphate only, and in 3 nodules that receive electric pulses only, there was no response observed. This study clearly showed antitumor effects of *EpECT* in metastatic melanoma. Although it not achieved a 100 % cure, it may be an effective alternative to palliative surgery or irradiation in these patients.[180]

Five cancer centres have performed an independent clinical trial with 20 malignant melanoma patients with 142 nodules treated with electro-pulse enhanced chemotherapy, and the objective responses of treatment the metastatic malignant melanoma nodules is estimate to be 95%.[181] In 57 % of the treated tumours, the tumour rapidly disappeared within 1-2 weeks after treatment which resulted in a complete response, and no regrowth of these nodules observed. The observed partial response was 38 %, and 3.5 % showed stable disease. In two cases, however, progress of the disease was observed.[31]

Bleomycin 10 mg/m^{2}BSA administered intravenously in eight patients with cutaneous metastases in malignant melanoma enrolled in another clinical study in France. Four patients received 4 pulses per nodule at an amplitude of 1000V per cm of electrode distance, and another four patients received 8 pulses at 1300V per cm of electrode distance. The result of the 1000V/cm exposure group, with an average tumour size of 6 mm, was 14% complete remissions and 86% partial remissions. In the group of 1300 V/cm, with average tumour size of 15 mm, there was 8 % complete remissions and 84% partial remissions, while 8% experienced stable disease.[182]

Bleomycin *EpEChT* of a patient with haemorrhaging malignant melanoma skin metastases in Denmark led to an effective and immediate relief of symptoms. Treatment of the 9 skin metastases, of which seven were ulcerated developed crusts, and the lesions healed in a matter of weeks. Thus Bleomycin electro-pulse enhanced chemotherapy could be considered for the palliation of haemorrhaging metastases as it is an efficient and tolerable treatment.[183,184]

From November 1998 through to November 1999, 15 patients with 38 skin lesions participated in phase II prospective clinical trial. There were 2

patients with 13 nodules of malignant melanoma (MM) treated with intra-lesional Bleomycin plus electric pulses at a field strength of 1300 V/cm and 100 μs pulse-length delivered 10 min after Bleomycin injection at a frequency of 1 Hz. After a follow-up of about 9 months there was 84.5 % objective response, with 23% complete response, 61,5 % partial response and 15.3 % stable disease.[158]

Nineteen patients with metastatic melanoma were enrolled in a phase two, randomized, open-label study comparing intra-lesional Bleomycin + EPT, and intra-lesional Bleomycin alone. Of 18 study lesions, 13 (72%) showed a complete response, one (5%) showed a partial response, three (18%) showed no change and one (5%) showed disease progression over a period of more than 12 weeks.[185,186]

A case of electro-pulse enhanced chemotherapy with Bleomycin, of multiple small unresectable cutaneous melanoma metastases on the thigh of a 59-year-old woman, resulted after 4 treatment sessions in a complete response of 224 treated melanoma nodules.[187]

In a study of treatment of subcutaneous melanoma metastases with electro-chemotherapy, seven patients with 81 cutaneous were treated under general anaesthesia using intravenous Bleomycin injection. After 218 days' follow-up, the objective response was 68%, with 25% complete response and 43% partial response. No response appeared in 26% of the patients, and 6% experienced progressive disease.[188]

In the following case series, the clinical effectiveness of *EpEChT* was studied as an alternative palliative treatment option for unresectable metastatic lesions of malignant melanoma with a systematic review of reported outcomes. Nine patients with 158 cutaneous and subcutaneous metastases, were treated with ECT under general anaesthesia using intravenous Bleomycin injection. After a follow-up time of 195 days, the fraction of complete response was 23%, and partial response was 39%. No response recorded in 30% and 8% of the cases experienced progressive disease.[189]

In a retrospective study, clinical features, treatment response, and side effects evaluated in 56 patients treated with electro-pulse enhanced chemotherapy at 6 German dermatology departments. The mean age of the patient cohort (14 men, 42 women) was 69.3 years. The fraction of complete response was 20%, partial response was 30%, and no response recorded in 20% of cases. The objective response was 50%, but as much as 30% of the cases experienced progressive disease.[190]

Thirteen cancer centres participated in the "*International Network for Sharing Practices on Electro-pulse enhanced chemotherapy with intra-tumoural or intravenous injection of Bleomycin under local or general*

anaesthesia," studied in total, 151 patients with metastatic melanoma. The study included 114 who had follow-up data of 60 days or more. Treatment of these patients with 394 lesions for 60 days resulted in an objective response of 78%, with 58% complete response, and 20% partial response. A follow-up of the 114 evaluable patients after more than 60 days, resulted in an objective response of 74%, with 48% complete response, and 26% partial response. Stable disease observed in 23% of the patients, and 3% experienced disease progression.[191]

Table 6-1 shows the data of the response of Electro-pulse enhanced chemotherapy treatments of Malignant Melanoma nodules of various sizes reported in 2017 by Kunte et al.[191]

Table 6-1
Response of Electro-pulse enhanced chemotherapy treatments of Malignant Melanoma nodules of various sizes.[191]

Tumour Volume	f_{CR}	f_{PR}	f_{SD}	f_{PD}	OR	WR_3	WR_4
5	0.68	0.11	0.21	0.00	0.79	0.72	0.79
8	0.64	0.15	0.21	0.00	0.79	0.69	0.77
16.5	0.56	0.18	0.25	0.01	0.74	0.61	0.70
25.5	0.34	0.44	0.20	0.02	0.78	0.47	0.59
30	0.38	0.38	0.21	0.03	0.76	0.48	0.59

There seems to be no variation in the objective response due to the size of the lesion, while as seen in Figure 6-4 the weighted responses WR_3 and WR_4 seem to follow a "dose-response functional relation with the size of the lesion (LS).

The decrease the weighted response is mainly due to the increase of progressive disease for patients with nodules of larger size.

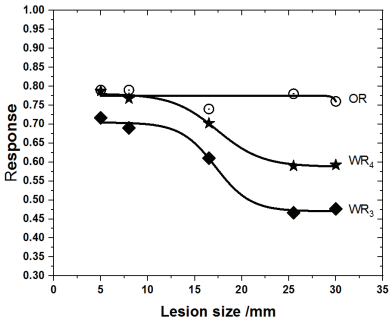

Figure 6-4
Objective response OR and Weighted responses WR_3 and WR_4 of Electro-pulse enhanced chemotherapy treatments of Malignant Melanoma nodules of various sizes. The data of Table 6-1[191] are fitted to dose response equation.

Summary of Malignant Melanoma

Table 6-2 shows a summary of the results of treatment Malignant Melanoma with electro enhanced chemotherapy reviewed in this section.

Table 6-2
Responses of Malignant Melanoma treatment with Electro Enhanced Chemotherapy using various devices.

Device Author[Reference]	No. Pat	No. Tum	f_{CR}	f_{PR}	f_{SD}	f_{PD}	OR
BTX (USA) Medpulser							
Heller,1996 [179]	5	23	0.78	0.17	0.05	0.00	0.95
Glass,1996 [180]	3	18	0.25	0.22	0.42	0.00	0.47
Byrne,2005 [186]	19	18	0.72	0.05	0.18	0.05	0.77
HM (Mexico)							
Rodriguez-Cuevas, 2001[158]	2	13	0.23	0.62	0.15	0	0.85
Juan (France)							
Mir,1998[31]	20	142	0.57	0.38	0.04	0.01	0.95
Mir,1998[31]	20	142	0.53	0.39	0.08	0.01	0.92
Rols,2000[182]	4	51	0.08	0.84	0.08	0	0.92
Cliniporator IGEA(Italy)							
Gehl,2000[183]	1	9	1.00	0.00	0.00	0.00	1.00
Snoj,2007[187]	1	224	1.00	0.00	0.00	0.00	1.00
Kis,2010[188]	7	81	0.25	0.43	0.26	0.06	0.68
Kis,2011[189]	9	158	0.23	0.39	0.30	0.08	0.62
Kreuter,2015[190]	20	20	0.20	0.30	0.20	0.30	0.50
Kunte,2017[191].	151	506	0.58	0.20	0.20	0.02	0.78
Kunte,2017[191].	114	394	0.48	0.26	0.23	0.03	0.74
Sum/Average	376	1799	0.49	0.30	0.16	0.04	0.80
/SD		14	0.29	0.23	0.12	0.08	0.17
/ SE			0.08	0.06	0.03	0.02	0.05

Table 6-3
The objective responses and all weighted response of treating malignant Melanoma, with different devices

Device	OR	SD	WR_3	SD	WR_4	SD
Medpulser	0.73	0.14	0.62	0.15	0.69	0.12
HM, Mexico	0,85		0.44		0.58	
Juan(France)	0.3	0.01	0.56	0.10	0.67	0.08
Cliniporator	0.76	0.07	0.54	0.14	0.62	0.12
All Average	0.80	0.05	0.55	0.07	0.64	0.06

Figure 6-5 shows the objective responses OR and weighted responses WR_4 of treatment Melanoma with various devices. The overall average is shown at the top of the figure.

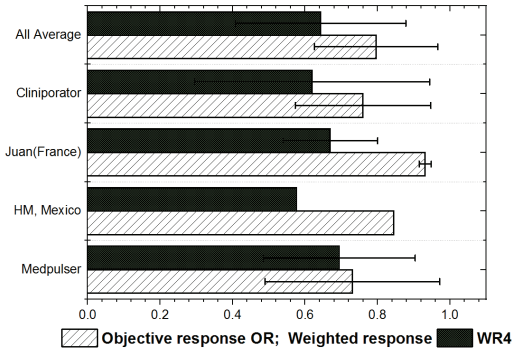

Figure 6-5
The objective response OR and weighted response of treatment Melanoma with various devices. The overall average is shown at the top of the figure.

6.1.3. SQUAMOUS CELL CARCINOMA

The first clinical trial of electro-pulse enhanced chemotherapy was on 8 patients with 42 nodules of head and neck squamous cell carcinoma.[28] There were 55% complete remissions and 14% partial remissions. Thus the objective response was 69%, and in 31 % there was no response.[28,156]

A phase I clinical trial performed with patients with head and neck squamous cell carcinoma (HNSCC) permeation nodules, treated with electro-pulse enhanced chemotherapy after intravenous injection of 10-15mg Bleomycin per m^2BSA. Of the ten evaluable patients, there were 11% with complete remissions and 11% with partial remissions. In 56% there was no response, and 22% experienced progressed disease.[192,193]

In one study of electro-pulse enhanced chemotherapy of cutaneous and subcutaneous tumours using intra-lesional Bleomycin, a single SCC a tumour achieved a partial remission.[32]

Independent clinical trials of electro-pulse enhanced chemotherapy performed by five cancer centres involved 17 patients with a total of 87 head and neck nodules of Squamous Cell Carcinoma, and resulted in complete responses appeared in 43 % and partial response in 19% which give an objective response of 62%.[31]

Electro-pulse enhanced chemotherapy with an intra-tumoural injection of Bleomycin of one patient with a single nodule of squamous cell carcinoma resulted in partial response.[194]

A phase II prospective clinical trial took place during 1998-1999 that involved two patients with 2 nodules of squamous-cell carcinomas (SCC). Intra-lesional Bleomycin administered according to Figure 6-1, and 10min after Bleomycin injection, electric pulses at a field strength of 1300 V/cm and pulse-length of 0.1ms were delivered at a frequency of 1Hz. The pulse generator and the needle array electrodes were especially designed and constructed by the Biomedical Engineering Department at *Instituto Mexicano del Seguro Social (IMSS), Mexico City, Mexico.* After a follow-up of about 9 months, there appeared no complete response and 100% partial response.[158]

Another study within the framework of a European clinical trial of electro-chemotherapy included 12 patients with oral squamous cell carcinoma. After treatment, ten patients were free of a tumour, and 2 patients had signs of SCC left, while one patient died due to neck metastasis. Thus the objective response was 100%, with 83% complete remissions and 17 % partial remission, while there was 8% progressive disease.[195]

A different clinical study looked at the effect of electric pulse`s duration and number in a sequence for electro-pulse enhanced chemotherapy of

patients with spindle-squamous cell carcinoma, One group exposed to 16 biphasic pulses of 25+25μs duration, which resulted in 71.4% complete remissions and 28.6% progressive disease, while another group exposed to 50+50μs biphasic pulses resulting in 78.6% complete remissions and 21.4 % progressive disease.[196]

Within a multicentre, single-arm Phase II study of electro-chemotherapy treatment, 54 patients were included with 69 squamous-cell carcinoma tumours of the head and neck. The treatment resulted in an objective response of 57%, with 25% complete remissions, 32 % partial remission, while 43% experienced no response. Eight patients were treated with Bleomycin alone with no clinical response and progressive disease. Thus electro-pulse enhanced chemotherapy with Bleomycin had a significant ($p < 0.001$) clinical response compared to Bleomycin alone.[197]

A clinical trial to determine the safety and effectiveness of Bleomycin electro-pulse enhanced chemotherapy was performed in two patients with 17 tumours in the head and neck. Complete remissions appeared in 82.4 %, and partial remissions in 17.6 % of the treated tumours.[198]

From 2004 to 2006, a total of 26 patients with oral squamous-cell carcinoma (OSCC) were treated with electro-pulse enhanced chemotherapy according to the ESOPE protocol. After 6-23 months of follow up, there was an objective response of 88% with 85% complete remissions and 4% partial remissions, while 12% experienced progressed disease. Most patients experienced pain as a complication which required high-dose pain medication for up to eight weeks.[199]

A study was performed between August 2003 and June 2005, in thirty patients with 111 nodules of various types treated with electro-pulse enhanced chemotherapy at the Mercy University Hospital, Cork. In five patients with 5 nodules of squamous-cell carcinoma, there was an objective response of 67 %, with 33% complete remissions. In 2 patients with 3 nodules < 3 cm, the objective response was 22 % with 11 % complete remissions.[200]

In a report of the clinical outcome from a single institution's experience with 52 patients, one case of squamous-cell carcinoma with 31 nodules > 30mm was involved. The authors, however, did not specify the treatment results quantitatively.[201]

In a clinical study of 15 patients with Head and Neck Cancers were nine cases of squamous-cell carcinoma involved. The authors, however, did not specify the treatment results of those cases quantitatively.[202]

In a clinical study, 6 patients with skin cancer of the head and neck were treated using electroporation therapy with intra-tumorally injected Bleomycin. After 4 weeks follow up the single patient with SCC of the

external meatus of the auditory canal, experienced persistent SCC with no response to a second treatment.[203]

A clinical study of primary T1 and T2 oral tongue cancer, included 15 patients of whom 14 had squamous cellular carcinoma. The objective response was 71.4% complete remissions while 38.6% experienced progress disease.[204]

A clinical electro-pulse enhanced chemotherapy phase II study took place as a collaboration between two centres, one in Denmark and the other in the UK. The study included fifty-two patients with cutaneous metastases of any histology of whom there were 3 with squamous-cell carcinoma. The authors, however, did not specify the treatment results of those cases.[205,206]

A case of an 80-year-old male with multiple erythematous nodules at the right *fronto-parieto-occipital* region was treated with electro-pulse enhanced chemotherapy with Bleomycin 15 mg/m^2 i.v., by using the Cliniporator™ device (IGEA Ltd.). At week 2 after treatment, there was a complete response. At week 8, however, a relapse occurred and a second *EpEChT* treatment performed. New complete regression appeared, but the patient died 3 months later of a systemic progression.[207]

In Greece, five medical centres performed electro-pulse enhanced chemotherapy according to the ESOPE protocol in 52 patients with a variety of tumours. Fourteen patients with Head and Neck tumours (very likely SCC) showed an objective response to the treatment of 93% with 64% complete remissions and 29% partial remission, while 7% experienced stable disease.[208]

From April 2009 to January 2011, a clinical study of electro-pulse enhanced chemotherapy was performed with a total of 15 patients with head and neck cancers, of whom 13 with squamous-cell carcinoma. After a follow-up in early 2012, the treatment 28 nodules resulted in an 86% objective response with 50% complete remissions and 36% partial remissions. Stable disease occurred in 4%, and 11% experience progressive disease.[209]

A clinical study focused on the role of electro-pulse enhanced chemotherapy in the treatment of 25 patients with head and neck cutaneous and subcutaneous cancers, of whom 13 had squamous-cell carcinoma. Treatments were performed using i.v. Bleomycin and the ESOPE protocol and after about 22 months, the treatment result was 100% objective responses, with 62% complete remissions, and 38% partial remissions.[210]

A preliminary study of electro-pulse enhanced chemotherapy of head and neck cancer in 8 elderly patients with 28 nodules of squamous-cell carcinoma, resulted in 50% complete remissions and 30% partial remissions.[211]

A meta-analysis was conducted to investigate the differences in the effectiveness of ECT concerning tumour type, drug, and route of administration. The overall objective response of electro-pulse enhanced chemotherapy treatment across all studies included in the analysis was estimated to 84.1%. The analysis, however, did not consider the progress of disease after treatment.[172]

A man affected by an ulcerated SCC and extensive metastasis was treated with electro-pulse enhanced chemotherapy with excellent clinical results, although all other treatment approaches rejected.[212]

A clinical study of Electro-pulse enhanced chemotherapy for cutaneous and subcutaneous metastasis was performed in 39 patients, of whom 3 were squamous-cell carcinoma, The authors, however, did not specify the treatment results of the SCC cases.[213]

In another clinical study, eight patients with recurrent or persistent squamous cell cancer in the head and neck area were treated with electro-pulse enhanced chemotherapy. The objective treatment response was 78%, with 33.3% complete remissions and a 44.4 % partial response, while in 22.2% there was progression of the disease.[214]

A retrospective study of 12 patients with 24 nodules of Squamous Cell Carcinoma (SCC) of the oral cavity or oropharynx treated with Bleomycin electro-pulse enhanced chemotherapy, resulted in an objective response of 50%, with 25% complete remissions and 25% partial remission, while 46% experienced stable disease, and 6% progression of the disease.[215]

In a clinical electro-pulse enhanced chemotherapy study of patients with primary SCC T2 cancer of the oral cavity or oropharynx performed with intratumoural administered Bleomycin, control biopsies were carried out 2 months after treatment. Complete remission experienced in all 4 patients but complications of bleeding, osteonecrosis and a fistula occurred.[216] Long-term (25-57 months) follow of 18 patient with Squamous Cellular Carcinoma treated with electro-pulse enhanced chemotherapy resulted in 78% complete remissions, while 23 % experienced progress disease.[217]

A prospective study of electro-pulse enhanced chemotherapy according to the ESOPE protocol followed by conventional chemo-radiotherapy was conducted among four patients with squamous cell carcinoma of the oral cavity in advanced stages. After 4 weeks 75% showed a partial response, and in 25% there was stable disease.[218]

In a retrospective clinical study, 56 patients with the various types of tumours treated with electro-pulse enhanced chemotherapy at 6 German dermatology departments. Mean age of the patient cohort (14 men, 42 women) was 69.3 years. The “carcinoma “tumour group included 15 patients of whom five had primary squamous-cell carcinomas of the skin,

three had Merkel cell carcinomas, two had vulvar carcinomas, and five had carcinomas of the head and neck region. The objective response in this group was 20%, with 6.7% complete response and 13.3% partial response, while 80% experienced progressive response of their carcinomas.[190]

A clinical study involving 22 patients with squamous-cell carcinoma aimed to evaluate pain scores before and after electro-pulse enhanced chemotherapy showed that the majority of patients had no or mild pain after electro-pulse enhanced chemotherapy.[219]

Between 2008 and 2013, a multi-centre study with 10 clinical centres study enrolled 41 patients with squamous-cell carcinoma for electro-pulse enhanced chemotherapy with intravenous administration of Bleomycin. The objective treatment result was 85.2% with 40.7% complete remissions and 44.5% partial remissions, while 14.8% experienced stable disease.[220]

A three-centre prospective phase II electro-pulse enhanced chemotherapy trial involved 25 patients with 35 nodules of squamous-cell carcinoma. The objective treatment result was 80% with 52% complete remissions and 28% partial remissions, while 20 % experienced stable disease.[221]

A European multi-institutional prospective study of electro-pulse enhanced chemotherapy performed according to the ESOPE protocol, involved 50 patients with squamous-cell carcinoma of whom 46 were evaluable. The objective treatment result was 80.4% with 56.5% complete remissions, and 23.9 % partial remissions, while 15.2% experienced stable disease and 4.3% progress of the disease.[222]

A retrospective single-centre study was performed to evaluate electro-pulse enhanced chemotherapy efficacy in the treatment of locally advanced stage III cutaneous squamous-cell carcinoma in 22 patients. The treatment followed the ESOPE guidelines, and after a mean follow-up 4 weeks, the objective response (according to *Table* 1) was 77%, with 22.7% complete responses and 54.5% partial responses, while 14 % experienced no response and 9 % progression of the disease.[223]

The D-EECT™ protocol, with successively decreasing voltage for each pulse, was recently applied in a clinical case study in India.[178] The study involved 8 patients with head *&* neck squamous cell carcinoma, a Bleomycin dose of 15 USP units/m^2 was administrated intra-tumorally i.t., or intravenously i.v. The objective response was 100%, with 15% complete response and 85% partial response, and none with stable or progress disease.

Plaschke (2019), presented the *DAHANCA 32* study, that is a phase II clinical trial in 26 patients with recurrent head and neck carcinomas with no curative treatment options. Electrochemotherapy was performed according to the ESOPE protocol under general anaesthesia with no serious adverse

events occurred during treatment. The tumour response was evaluated by using CT scanning, MRI and ^{18}FDG-PET as well as biopsy results and questionnaires about, safety, toxicity, pain score, and quality-of-life. In the follow-up at week-4 follow-up of the 26 patients treated of whom 22 were evaluated resulted in 18% CR, 36% PR, 41% SD and 5% PD which gave an objective response OR of 55%. In the follow-up week-8 were 25 patients evaluated which resulted in 20% CR, 40% PR, 36% SD and 4% PD, which resulted in an objective response OR of 60%. Two responders remain without recurrence. Four events occurred post treatment: one bleeding episode, two episodes with mucosal swelling, and one patient died due to disease progression.[224]

Table 6-4

The number of cases, and clinical fractional treatment response 'f-TR' at week 4, and week 8 follow-up after the treatment. [224]

Response	Week 4 No. cases	Week 8 No. cases	Week 4 f-TR	Week 8 f-TR
CR	4	5	0.18	0.20
PR	8	10	0.36	0.40
SD	9	9	0.41	0.36
PD	1	1	0.05	0.04
SUM	22	25	1.00	1,00
Not Applicable	3	0		
Tot Sum	25	25		
OR			0.54	0.60

Before treatment, and at follow-up at week4 and week 8, the tumour expanse evaluated by CT using the longest diameter of the entire tumour according to RECIST criteria version 1.1. The tumour expanse values` TX was evaluated by using MR expanse area TA**,** and by using **PET s**tandardized uptake values (SUVs).

The specific therapeutic response (STR_X) of the treatment was estimated as the difference between the untreated "UT" and treated "T" total tumour expanse "TX", as recorded by CT, MRI or PET, divided by the expanse of untreated tumour:

$$STR_x(t) = \frac{TX^{UT} - TX^{T}(t)}{TX^{UT}} \qquad \text{Eq. 6.5}$$

where

x = expanse recorded by x = CT, MRI or PET respectively
$STR_x(t)$ = Specific Therapeutic Response at follow-up time t
TX^{UT} = Tumour expanse of untreated "UT" tumour as recorded by x
$TX^{T}(t)$ = Tumour expanse of treated "T" tumour at follow-up time t

The STR is equal to 1 when, $TX^T = 0$ which indicate CR
The STR is less than 1 when, $0 < TX^T < TX^{UT}$ which indicate PR
The STR is about 0 when, $TX^T \approx TX^{UT}$ which indicate SD
The STR is less than 0, when , $TX^T > TX^{UT}$ which indicate PD

Response evaluated by CT imaging

The specific therapeutic response $STR_{CT}(t)$, was evaluated by using the values of CT-RECIST (cm/response) given in *Table*-3 of the publication.[224] The Figure 6-6 shows the specific therapeutic response at week 4 after treatment $STR_{CT}(4)$, and the Figure 6-7 shows the specific therapeutic response at week 8 after treatment $STR_{CT}(8)$.

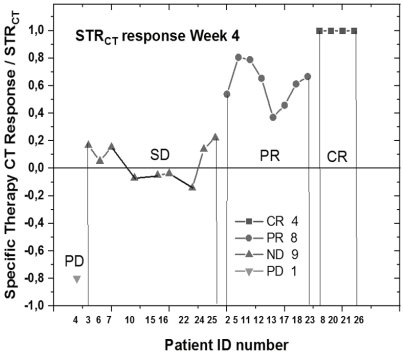

Figure 6-6
The Specific Therapeutic Response as recorded by CT at week 4 after treatment.

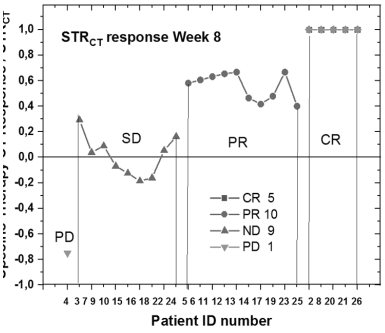

Figure 6-7
The Specific Therapeutic Response as recorded by CT at week 8 after treatment.

The STR_{CT} values for stable disease SD-cases are significantly different from the progressive response PR-cases ($p = 10^{-6}$) which are significantly different from the complete response CR-cases. Thus CT scanning seems to be a relevant method for objective evaluating the treatment response of electro enhanced chemotherapy.

Table 6-5
The clinical treatment response "TR", longest diameter "d^{CT}" and specific therapy response "STR_{CT}" as recorded by CT, at week 4 and week 8 follow-up.

Pat. No.	TR[1)] w4	TR[1)] w8	d^{CT} mm w0	d^{CT} mm w4	d^{CT} mm w8	STR_{CT} w4	STR_{CT} w8
2	PR	CR	3.9	1.8	0	0.54	1.00
3	SD	SD	2.4	2	1.7	0.17	0.29
4	PD	PD	2	3.6	3.5	-0.80	-0.75
5	PR	PR	3.1	0.6	1.3	0.81	0.58
6	SD	PR	3.8	3.6	1.5	0.05	0.61
7	SD	SD	5.2	4.4	5	0.15	0.04
8	CR	CR	1.7	0	0	1.00	1.00
9	SD	SD	4.5		4.1		0.09
10	SD	SD	2.9	3.1	3.1	-0.07	-0.07
11	PR	PR	1.9	0.4	0.7	0.79	0.63
12	PR	PR	2.6	0.9	0.9	0.65	0.65
13	PR	PR	2.7	1.7	0.9	0.37	0.67
14	SD	PR	2.8		1.5		0.46
15	SD	SD	4	4.2	4.5	-0.05	-0.13
16	SD	SD	2.7	2.8	3.2	-0.04	-0.19
17	PR	PR	2.4	1.3	1.4	0.46	0.42
18	PR	SD	3.1	1.2	3.6	0.61	-0.16
19	SD	PR	2.3		1.2		0.48
20	CR	CR	3	0	0	1.00	1.00
21	CR	CR	2.2	0	0	1.00	1.00
22	SD	SD	5.6	6.4	5.3	-0.14	0.05
23	PR	PR	4.8	1.6	1.6	0.67	0.67
24	SD	SD	4.3	3.7	3.6	0.14	0.16
25	SD	PR	4.5	3.5	2.7	0.22	0.40
26	CR	CR	2.4	0	0	1.00	1.00
Average						0.39	0.40
SD						0.47	0.46

[1)]The weights` w_{CR}=3, w_{PR}=1, w_{SD}=0 and w_{PD}=-3 are used in the numerical recording of the clinical response in *Table* 2 used for PCA

Table 6-6
Summary of the clinical treatment response "TR", longest diameter "d^{CT}" and specific therapy response "STR_{CT}" as recorded by CT, at week 4 and week 8 follow-up.

Clin. Resp	Ave. STR_{CT} Week 4	SD	No. w4	*f*-TR w4	Ave. STR_{CT} w8	SD	No. w8	*f*-TR w8
PD	-0.80		1	0.05	-0.75		1	0.04
SD	0.05	0.13	9	0.41	0.01	0.16	9	0.36
PR	0.61	0.15	8	0.36	0.56	0.11	10	0.40
CR	1.00	0.00	4	0.18	1.00	0.00	5	0.20
ND	-	-	3					
Ave Sum	**0.39**	0.47	22+3	1	**0.40**	0.46	25	1
WR_4	**0.42**				**0.45**			

The values of weighted response WR_4 evaluated from the clinical response fraction values in *Table*-3 are 0.42 at week 4 and 0.45 at week 8. These values correspond very well with the averages of specific treatment response STR_{CT} evaluated with CT examination.

Figure 6-8 and Figure 6-9 shows the result of principal component analysis of the data given in *Table* 2. The three encircled groups represent the complete remissions, partial remissions and Stable disease. The figures show that the case No. 4 with progressive disease is an outsider, well separated from the others. The clinical response (TR) coincides with a specific therapeutic response STR_{CT}, and there is no correlation with tumour expanse CT_0 before treatment.

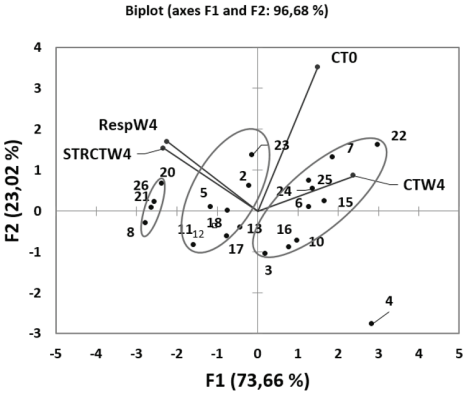

Figure 6-8
PCA-biplot of the CT response at week 4 follow up

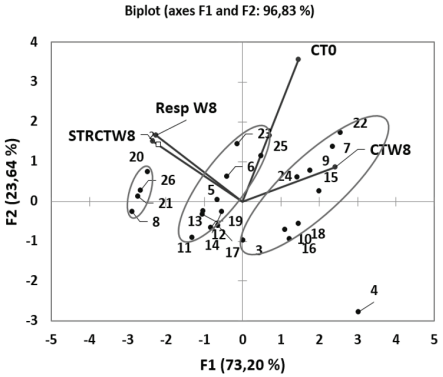

Figure 6-9
PCA-biplot of the CT response at week 8 follow up

Response evaluated by MR imaging

The specific therapeutic response $STR_{MR}(t)$ was evaluated by using the values of expanse treatment area evaluated measured on MRI from two perpendicular measurements given as "MRI (cm^2 response)" in *Table* 3 of the publication.[224]

The Figure 6-10 shows the specific therapeutic response $STR_{MR}(4)$ at week 4 after treatment, and the Figure 6-11 shows the specific therapeutic response $STR_{MR}(8)$ at week 8 after treatment.

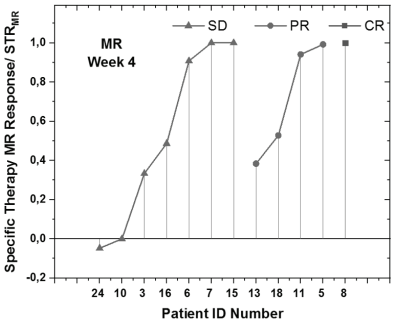

Figure 6-10
The Specific MR Therapeutic Response at week4 after treatment

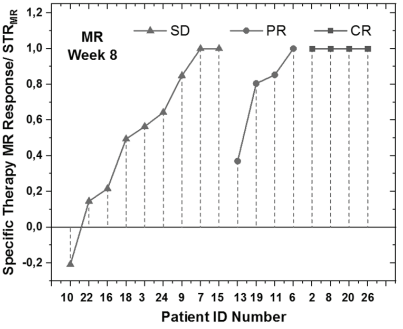

Figure 6-11
The Specific MR Therapeutic Response at week 8 after treatment

The STR_{MR} values for stable disease SD-cases are not significantly different from partial response PR-cases (p=0.4) which are not significantly

different (p=0.24) from CR cases. Thus MR scanning seems not to be a relevant method for objective evaluating the treatment response of electro enhanced chemotherapy.

Response evaluated by PET imaging

Standardized uptake values (SUVs) are used in clinical FDG-PET oncology imaging, and has a specific role in assessing patient response to cancer therapy. *Positron Emission Tomography Response Criteria in Solid Tumours* (PERCIST 1.0) were introduced in 2009 as guidelines for systematic and structured assessment of response to therapy with ^{18}F-deoxy-glucose (FDG) PET in patients with cancer, with suggested application in clinical trials and, potentially, in the clinical practice of PET reporting.[225]

In the study[224] the SUVpeak is measured at baseline in the interior tumour using PERCIST. By week 4 and 8, SUV-peak values are evaluated by using a sphere of at least one cm^3 within the treatment area. The specific therapeutic response $STR_{PET}(t)$ was evaluated by using the values of "*PET - SUV in (SU Vpeak/response)*" given in *Table* 3 of the publication [224]. The Figure 6-12 shows the specific therapeutic response $STR_{PET}(4)$ at week 4 after treatment, and the Figure 6-13 show the specific therapeutic response $STR_{PET}(8)$ at week 8 after treatment.

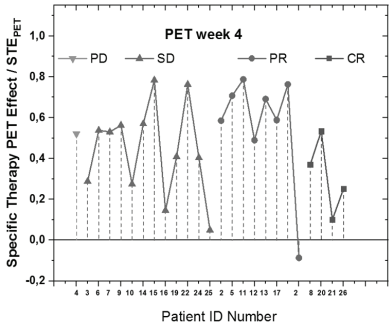

Figure 6-12
The Specific PET-SUV Therapeutic Response at week4 after treatment

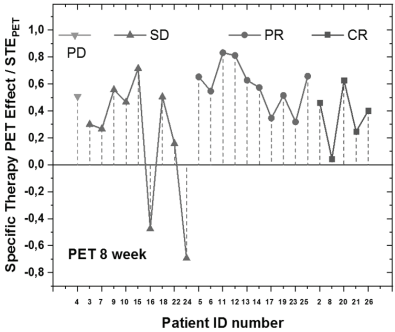

Figure 6-13
The Specific PET-SUV Therapeutic Response at week 8 after treatment

At the week 4 follow- up the STR_{PET} values for the stable disease SD-cases are not significantly different from the progress disease PR-cases (p=0.32) which are not significantly different (p=0.1) from the complete response CR-cases. Thus FDG-PET scanning seems not to be a relevant method for objective evaluating the early treatment response of electro enhanced chemotherapy.

At the week 8 follow up the STR_{PET} values for the stable disease SD-cases are significantly different from the partial response PR-cases (p=0.0,04). Although they are not significantly different (p=0.08) from the complete remission CR-cases. Thus FDG-PET scanning seems not to be a quite relevant method for objective evaluating the late treatment response of electro enhanced chemotherapy.

Summary of Squamous cell carcinoma

A summary of the results of treatment Squamous Cell Carcinoma with Electro Enhanced Chemotherapy reviewed in this section is given in Table 6-7.

Table 6-7
Summary of the results of treatment Squamous Cell Carcinoma with Electro Enhanced Chemotherapy using various types of devices

Device Author[Reference]	No. Tum.	No. Pat.	fCR	fPR	fSD	fPD	OR
BTX(USA) Medpulser							
Heller,1998[32]	1	1	0	1.00	0	0	1.00
Heller,1999[194]	1	1	0	1.00	0	0	1.00
Allegretti,2001[226]	12	12	0.50	0.21	0.29	0	0.71
Burian,2003[195]	12	12	0.83	0.08	0	0.08	0.92
Bloom,2005[197]	69	54	0.25	0.32	0.43	0.	0.57
Burian,2007[199]	26	26	0.85	0.04	0	0.12	0.88
Tijink,2006[198]	17	2	0.82	0.18	0.	0	1.00
Landstrom,2010[203]	6	5	0.33	0.33	0.17	0.17	0.67
Landstrom,2011[204]	14	14	0.71	0	0	0.29	0.71
Landstrom,2015[216]	4	4	1.00	0	0.	0	1.00
Landstrom,2015[217]	18	18	0.78	0	0	0.22	0.78
HM Mexico Rodriguez-Cuevas,2001[158]	13	2	0.00	1.00	0	0	1.00
S15 Jouan, (France)							
Mir, 1991[103]	42	8	0.55	0.14	0.31	0.00	0.69
Domenge,1996[193]	10	7	0.11	0.11	0.56	0.22	0.22
Mir, 1998[31]	77	17	0.43	0.19	0.27	0.10	0.62

ESOPE, IGEA Cliniporator							
Marenco,2011[207]	1	1	0	0	0	1.00	0.00
Skarlatos,2011[208]	14	14	0.64	0.29	0.07	0	0.93
Gargiulo,2012[210]	13	13	0.62	0.38	0.00	0	1.00
Mevio,2012[209]	28	12	0.50	0.36	0.04	0.11	0.86
Benevento,2013[211]	8	28	0.50	0.30	0.10	0.10	0.80
Seccia, 2014[214]	9	8	0.33	0.44	0.00	0.22	0.78
Campana,2014[215]	24	24	0.25	0.25	0.46	0.04	0.50
Domanico, 2015[218]	4	4	0.00	0.75	0.25	0.00	0.75
Kreuter,2015[190]	15	15	0.07	0.13	00	0.80	0.20
Campana,2016[220]	35	35	0.41	0.45	0.15	0.00	0.85
Rotunno,2016[221]	35	25	0.52	0.28	0.20	0.00	0.80
Bertino2016[222]	46	50	0.57	0.24	0.15	0.04	0.80
Di Monta,2017[223]	22	22	0.23	0.55	0.14	0.09	0.77
Plaschke, 2017[227]<3 cm	14	14	0.21	0.50	0.21	0.07	0.71
Plaschke,2017[227]>3 cm	20	20	0.20	0.40	0.30	0.10	0.60
Plaschke,2017[227]	34	34	0.21	0.44	0.26	0.09	0.65
Plaschke,2019[224]	22	26	0.18	0.36	0.41	0.05	0.54
Plaschke,2019[224]	25	26	0.20	0.40	0.36	0.04	0.60
IQWave, Chemotech							
Kalavathy, 2018[178]	12	8	0.15	0.85	0.00	0.00	1.00
Sum/Average	**562**	**669**	**0.38**	**0.35**	**0.15**	**0.12**	**0.73**
/SD		**12**	**0.28**	**0.28**	**0.16**	**0.21**	**0.24**
/SE		**3**	**0.05**	**0.05**	**0,03**	**0.04**	**0.04**

Comparison of the response of various devices

In the following Tables 6-8 to Table 6-9 the average treatment responses reported from clinical studies of squamous-cell carcinoma and breast cancers using different treatment devices are compared.

Squamous cell Carcinoma

- BTX Genetronix Medpulser[32,194,226,195,197,199,198,203,204,216,217]
- Home Made Mexican device[158]
- S 15 electropulsator, Jouan, (France)[103,193,31]
- ESOPE, Cliniporator IGEA (Italy)[207,208,210,209,211,214,215,218,190,220,221,222,223,227,224]
- D-EECT, IQWave Chemotech (Sweden)[178]

Table 6-8
The average clinical responses of treating SSC with different devices

Device/Protocol	No. Pat	No. Tum	fCR	fPR	fSD	fPD
BTX Medpulser USA	151	193	0.55	0.29	0.08	0.08
Home Made. Mexico	2	15	1.00	0	0	0
S 15 electropulsator. Jouan. (France)	32	105	0.36	0.15	0.38	0.11
Cliniporator IGEA. ESOPE	371	335	0.31	0.36	0.17	0.15
QWave Chemotech. D-EECT	8	12	0.15	0.85	0	0
Overall average	562	669	0.38	0.35	0.15	0.12

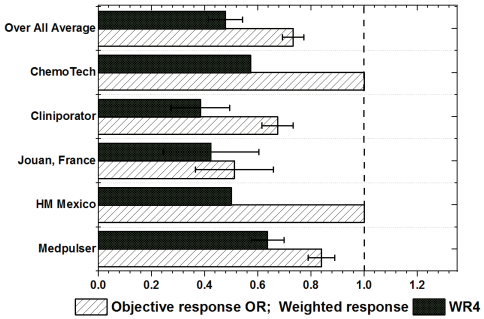

Figure 6-14
The objective responses and weighted response WR_4 of treating SSC with different devices
The overall average is shown at the top.

Table 6-9
The objective responses and all weighted response of treating SSC with different devices

Device/Protocol	OR	SE	WR_3	SE	WR_4	SE
BTX Medpulser USA	0.84	0.05	0.57	0.08	0.64	0.06
Home Made. Mexico	1		0.33		0.50	
S15 electropulsator. Jouan. (France)	0.51	0.15	0.30	0.20	0.42	0.18
Cliniporator IGEA ESOPE	0.67	0.06	0.28	0.11	0.38	0.11
IQWave Chemotech. D-EECT	1.00	0.14	0.43		0.57	0.12
Overall average	0.73	0.04	0.38	0.06	0.48	0.06

The BTX Genetronix Medpulser seems to deliver the best every weighted response WR_4=0.64 for SCC followed by IQwave WR_4=0.57, and the Home-Made Mexican device WR_4=0.5. The ESOPE protocol with Cliniporator had the lowest response WR_4=0.38. In those judgements, the sizes of tumours treated with the different devices are not considered. However, In the next paragraph, the reports with data of tumour size will be considered.

Figure 6-15 shows the Objective response of Squamous-cell carcinoma SCC of different tumour diameter treated with various types of devices. The Medpulser and IQwave seem both deliver 100% objective response, although the tumours treated with IQWave are twice as large. The Electroporator Jouan and Cliniporator seem both deliver an objective response of about 65%. Figure 6-15 shows the Weighted response WR_4 of Squamous cell carcinoma of different tumour diameter treated with various type of devices. In treatment of large tumours (diam.≈5cm) the IQWave-device deliver the best weighted response WR_4≈60%, while with tumours of corresponding size the weighted response of Cliniporator is WR_4≈40%. In treatment of small tumours (diam.<3cm) the weighted response WR_4 is about 45-50% for Cliniporator, Jouan and Medpulser.

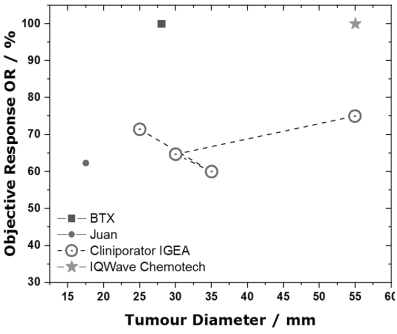

Figure 6-15
Objective response of Squamous cell carcinoma SCC treated with different devices
BTX Genetronix Medpulser[158], S15 electropulsator, (Jouan, France): [31], Cliniporator IGEA ESOPE[214, 227], IQWave D-EECT Chemotech[178].

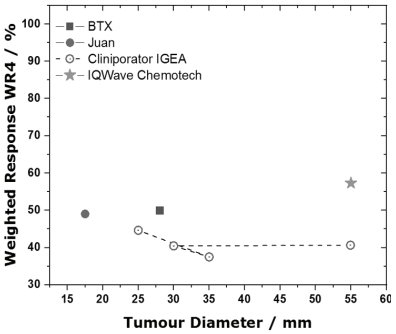

Figure 6-16
Weighted response WR_4 of Squamous cell carcinoma treated with different devices
BTX Genetronix Medpulser[158], S15 electropulsator, (Jouan, France): [31] [31] , Cliniporator IGEA ESOPE[214, 227], IQWave D-EECT Chemotech[178].

6.1.4 BREAST CANCER

Complete remission appeared in a patient with breast adenocarcinoma who received i.v. Bleomycin, and after 8-26 minutes, it was followed by 10 sessions of 4-8 short (0.1ms) pulses of 1000 or 1300V/cm, delivered between 8-26 minutes at adjacent positions on the nodule to cover all the tumour surface.[193]

A Phase I/II trial for the treatment of cutaneous and subcutaneous tumours using electro-pulse enhanced chemotherapy was initiated to determine if this mode of treatment would be effective against certain primary and metastatic cutaneous malignancies. The study enrolled a group of six patients, one with metastatic breast adenocarcinoma. The patient received a 15,000 IU dose of Bleomycin administered intravenously at a rate of about 1500 IU per minute. Five to 15 minutes after the Bleomycin thoroughly infused and the patient is injected with a 1% lidocaine solution at around the treatment site. The treatment was followed by eight short (99 μs) pulses at an amplitude of 1 300 V/cm administered directly to the tumours. The patient with metastatic breast adenocarcinoma showed complete responses in both treated nodules after *EpEChT*. The treatment tolerated well with no residual effects from the electric pulses. In conclusion the study indicated that *EpEChT* is an effective local treatment for metastatic breast and encourages further clinical studies.[179]

Five cancer centres have performed an independent clinical trial with a total of 291 cutaneous or subcutaneous tumours of which 2 were breast cancer with 10 nodules treated with electro-pulse enhanced chemotherapy. The tumours were measured, and the response to the treatment evaluated 30 days after the treatment. In the breast tumours treated with objective responses (OR) were recorded in only 2 nodules (20 %). The application of electric pulses to the patients was safe and well-tolerated.[31]

From November 1998 through to November 1999, 15 patients with 38 skin lesions participated in phase II prospective clinical trials, using intra-lesion Bleomycin (750-3500 IU). In the group of patients, there were two with totally 14 nodules of breast cancer. The treatment proceeds ten minutes after intra-tumour Bleomycin injection with six short electric pulses (0.1ms) delivered at a field strength of 1300V/cm, and with a frequency of 1Hz. The objective response of *EpEChT* treatment in this study was 100%, with 8 complete remissions (CR 58%) and 6 partial remissions (PR 42%). Although no complications appeared related to the treatment, more experience and longer follow-up were needed.[158]

A case report demonstrated that multiple applications of electro-chemotherapy could induce remission in a locally destructive breast cancer recurrence, which was refractory to other multimodal therapy.[168]

A two year long prospective non-randomised study in which four cancer centres enrolled patients evaluated the efficacy and safety of electro-pulse enhanced chemotherapy was carried out with Bleomycin or Cisplatin among 14 patients with breast cancer. The treatment of recurrent breast cancer performed out using intravenous or intra-tumour drug administration followed by application of electric pulses generated by a Cliniporator™ using plate or needle electrodes. In the 58 breast cancer nodules treated with electro-pulse enhanced chemotherapy, a complete response was recorded in 84 %, a partial response of 7 %, and stable disease of 10%, regardless of drug use or the route of its administration. The lack of detailed information makes it difficult to evaluate the outcome of this study further.[162]

Another study was performed to investigate the clinical utility of electro-pulse enhanced chemotherapy by treating 30 patients with cutaneous or subcutaneous recurrent tumours, which were inoperable, or progressive and refractory to systemic chemotherapy or radiotherapy. Of these patients, 12 had breast cancer with 99 nodules. After treatment with electro-pulse enhanced chemotherapy, 70 % of the breast tumours showed complete remission, 21% partial response and 9% no change.[200]

In a study of 52 consecutive patients with different type of cancer-histology, eleven were breast cancer, with 174 nodules treated with Bleomycin-based electro-chemotherapy. The treatment of breast tumours showed the objective response of 86%, with 50% complete remission, 36% a partial response and 14% with stable disease. None of the treated patients experienced a relapse in the treatment field. Thus *EpEChT* proved to be a safe, and effective treatment of breast cancer, that also preserves patients' quality of life.[201]

A phase II clinical study of electro-pulse enhanced chemotherapy engaged patients with cutaneous recurrent breast cancer nodules larger than 3 cm, for whom no further treatment options were available. The primary endpoint of the study was an objective response evaluated by clinical examination. Secondary endpoints included a response evaluated by PET/CT, a change in lung diffusion capacity, the patient reporting symptoms, and distress appearance. Treatment consisted of the i.v. injection of Bleomycin 15,000 IU/m^{2}BSA followed by an application of electric pulses according to the ESOPE protocol. Of the 12 evaluable patients with a follow-up >8 weeks, computed tomography CT showed that 33% of patients were achieving over 50% tumour volume reduction. The objective response was 25% with 8% complete remission, and 17% partial response.

As much as 70% experienced a stable disease, and 8% experienced a relapse in the treatment field.[228,206,229]

Another study of 12 elderly breast cancer patient with 142 cutaneous metastases with a diameter > 3cm in only 2 patients, was treated with Bleomycin electro-pulse enhanced chemotherapy according to the ESOPE criteria. The objective response was 92.3 %, with a complete response of 75.3%, a partial response of 17%, and no change in 7.7% of the nodules. From these results *EpEChT* recommends as a primary local therapy in patients, and was not deemed suitable for surgical removal of a primary tumour < 3cm, even in the elderly.[230]

Between 2010 and 2013 a multicentre electro-pulse enhanced chemotherapy study enrolled 125 patients with metastases of breast cancer, and treatment was performed according to the ESOPE protocol in 113 patients with 214 evaluable nodules. After 2 months' follow-up, there was an objective response of 90.2% with 58.4%, complete response, 32% partial response, while 7% experienced a stable disease, and 2% suffered a relapse of disease in the treatment field. At a one year's follow-up, 96.4% of the patients experienced durable local control. The fraction of complete remissions varied with the size of a tumour: 80.3 % CR for diam. < 3 cm, and 46.1 % CR for diam. >3 cm. Thus *EpEChT* represents a valuable therapy for breast cancer, particularly for patients with tumours < 3 cm diameter.[231]

In a retrospective study, clinical features, treatment response, and side effects of electro-pulse enhanced chemotherapy were evaluated in 56 patients with various diagnoses, and were treated according to the ESOPE protocol at six German dermatology departments. In a cohort which included thirteen patients with metastasized breast cancer, complete remission appeared in 8%, a partial response in 39%, and in 15% there was no change of tumour growth, while 39% experienced tumour progression.[190]

A case report presented a 61-year-old woman with unresectable breast cancer recurrence to the skin and subcutaneous tissue, for which numerous lines of treatment were unsuccessful. After three treatments with electro-chemotherapy, however, a dramatic regression of the cutaneous metastatic foci was recorded, and after an actual observation period of 12 weeks, complete clinical remission was achieved.[232]

Between 2010 and 2013 a total of 125 patients with breast cancer skin metastases were enrolled onto a multicentre retrospective cohort study of electro-pulse enhanced chemotherapy.

In Table 6-10 the various subgroups in the study are displayed for oestrogen receptor (ER) positivity, and low Ki67 index after treatment of

109 patients with a Cliniporator™ device, following the European Standard Operative Protocol (ESOPE). [233]

Table 6-10
Tumour response to electro-pulse enhanced chemotherapy according to surrogate definition of breast cancer intrinsic subtypes. [233]

Subtype	No.	CR %	PR %	SD %	PD %	NE %	OR %	WR_4 %
A-like: ER.PgR+	23	73.9	17.4	4.3	4.3	0	91	79
B-like: HER2-	22	50	40.9	9.1	0	4.5	75	73
B-like: HER2 +.	17	58.8	29.4	5.9	5.9	0	88	69
Triple-	35	57.1	31.4	11.4	0	0	89	76
HER2 +	11	54.5	45.5	0	0	0	100	77
Sum/ Ave. / *SD*	*108*	58.9 9	32.9 10.9	6.1 4.4	2 2.9	0.9 2	92 5	75 4

NE% is percentage of patients not included in the evaluation

At a patient follow up after two months, 59% of the whole breast tumour cohort showed complete remission, 31 % showed partial response and 7.4% a stable disease, while 1.9% experienced relapse in the treatment field. One tumour, was not applicable for evaluation.[233] The weighted responses derived according to equation 6.2 and 6.3 were 68% for WR_3, and 75% for WR_4 respectively.

Another study was a 10-year audit of a single centre experience of electro-pulse enhanced chemotherapy according to the European Standard Operating Procedures (ESOPE) with a Cliniporator™ device. In total, the patient cohort was 24 breast cancer patients who all had received prior multimodal therapy, and who had, or had developed 242 lesions. The objective response of *EpEChT* was 67 % with a complete response of 54 %, and a partial response of 13%, while 16 % experienced stable disease, and 1% experienced progressive disease. As smaller lesions were found to be more responsive, early treatment of *EpEChT* could apply within multimodal treatment strategies.[234]

A clinical study of seven breast cancer patients with 35 skin metastases treated with electro-pulse enhanced chemotherapy according to the ESOPE protocol resulted in 86% objective remissions, with 43% complete

remissions and 43% partial remissions, while 14 % experienced progressive disease. The results compare with the average results of 8 other published similar studies with a total average of objective response was estimated to 76% with 39 % complete remissions, while 16 % experienced stable disease and 8 % progressive disease.[235,177]

The results of the evaluation 90 patients with cutaneous recurrence from breast at two months after treatment with electro-pulse enhanced chemotherapy according to the ESOPE protocol at 10 European cancer centres, showed 74% objective remissions, with 52% complete remissions and 22% partial remissions, while 18% experienced stable and 8% progressive disease.[236] The only predictor variable considered related to the outcome of the treatment is the median diameter of the coetaneous metastases ranging from 1 to 550 mm. The results of the evaluable treatment of nodules <30 mm, and >30 mm summarized in Table 6-11a were derived from *table 5* in the publication of Matthiessen (2018).[236] Table 6-11b also shows the response parameters of the 83 evaluable patients at 2 months after the electro-pulse enhanced chemotherapy from *table 4* in the same publication.[236]

Table 6-11a

Tumour response to electro-pulse enhanced chemotherapy according to size of nodules < 30 mm. and > 30 mm. and at 2 months after treatment.[236]

	No. Pat.	No. Nodules	fCR	fPR	fSD	fPD	fNE
Nodules < 3cm		113	0.72	0.14	0.08	0.01	0.05
Nodules > 3cm		94	0.37	0.24	0.18	0.06	0.03
Average		207	0.57	0.20	0.13	0.03	0.04
Patients 2 months	90		0.52	0.22	0.18	0.08	0.03

fNE fraction of patients not included in the evaluation

Table 6-11b

The objective (fOR) and weighted responses fWR_3 and fWR_4 to electro-pulse enhanced chemotherapy according to size of nodules < 30 mm. and > 30 mm. and at 2 months after treatment.[236]

	No. Patients	No. Nodules	fOR	fWR_3	fWR_4
Nodules < 3cm		113	0.86	0.76	0.80
Nodules > 3cm		94	0.62	0.40	0.48
Average		207	0.76	0.60	0.67
Patients 2 months	90		0.74	0.51	0.60

Summary of Breast Cancer

A summary of the results of electro enhanced chemotherapy treatment of breast cancer with Bleomycin by using various devices is given in Table 6-12 and Table 6-13.

Table 6-12a

Tumour response to electro-pulse enhanced chemotherapy with Bleomycin of breast cancer by using various devices.

Device Author[Reference]	No. Pat.	No. Tum.	fCR	fPR	fSD	fPD
Medpulser, (USA)						
Heller,1996[179]	1	2	1.00	0.00	0.00	0.00
Larkin,2007[200]	12	99	0.70	0.21	0.09	0.00
Home Made (Mexico)						
Rodriguez-Cuevas,2001[158]	2	14	0.58	0.42	0.00	0.00
GTH 1287, Jouan (France).						
Domenge, 996[193]	1	1	0.00	1.00	0.00	0.00
Mir,1998[31]	2	10	0.20	0.00	0.80	0.00
Rebersek,2004[237]	6	12	0.33	0.42	0.25	0.00
Cliniporator IGEA (Italy) ESOPE						
Marty,2006[162]	14	58	0.84	0.07	0.10	0.00
Campana,2009 [201]	11	174	0.50	0.36	0.14	0.00
Matthiessen,2012[229]	12	12	0.08	0.08	0.75	0.08
Benevento,2012[230]	12	142	0.75	0.17	0.08	0.00
Cabula,2015[231]	112	214	0.59	0.32	0.07	0.02
Kreuter,2015[190]	13	13	0.08	0.38	0.15	0.38
Wichtowski,2016[232].	1	10	1.00	0.00	0.00	0.00
Bourke,2017[234].	24	202	0.64	0.15	0.19	0.01
Campana,2017[233]	108	239	0.59	0.31	0.07	0.02
Wichtowski,2017[177]	7	35	0.43	0.43	0.00	0.14
Matthiessen,2018[236]	90	207	0.52	0.22	0.18	0.08
IQWave ChemoTech (Sweden) D-EECT						
Kalavathy,2018[178]	5	11	0.40	0.60	0.00	0.00
Sum	433	1455				
Average			0.51	0.29	0.16	0.04
SD			0.30	0.25	0.24	0.09
SE			0.07	0.06	0.06	0.02

Table 6-12b

The objective (fOR) and weighted responses fWR_3 and fWR_4 to electro-pulse enhanced chemotherapy with Bleomycin of breast cancer by using various devices.

Device Author[Reference]	No. Patients	No. Nodules	OR	WR_3	WR_4
Medpulser, (USA)					
Heller,1996[179]	1	2	1.00	1.00	1.00
Larkin,2007[200]	12	99	0.91	0.77	0.83
Home Made (Mexico)					
Rodriguez-Cuevas,2001[158]	2	14	1.00	0.72	0.79
GTH 1287, Jouan (France).					
Domenge, 996[193]	1	1	1.00	0.33	0.50
Mir,1998[31]	2	10	0.20	0.20	0.40
Rebersek,2004[237]	6	12	0.75	0.47	0.60
Cliniporator IGEA (Italy)					
Marty,2006[162]	14	58	0.90	0.86	0.89
Campana,2009 [201]	11	174	0.86	0.62	0.72
Matthiessen,2012[229]	12	12	0.17	0.03	0.23
Benevento,2012[230]	12	142	0.92	0.81	0.86
Cabula,2015[231]	112	214	0.91	0.68	0.75
Kreuter,2015[190]	13	13	0.46	-0.18	-0.08
Wichtowski,2016[232].	1	10	1.00	1.00	1.00
Bourke,2017[234].	24	202	0.80	0.68	0.76
Campana,2017[233]	108	239	0.91	0.68	0.75
Wichtowski,2017[177]	7	35	0.86	0.43	0.51
Matthiessen,2018[236]	90	207	0.74	0.51	0.59
IQWave ChemoTech (Sweden)					
Kalavathy, 2018[178]	5	11	1.00	0.60	0.70
Sum	433	1455			
Average			0.80	0.57	0.66
SD			0.26	0.32	0.27
SE			0.06	0.08	0.07

Table 6-13

Summary of objective and weighted responses to electro-pulse enhanced chemotherapy with Bleomycin of breast cancer by using various devices.

Device	OR	SE	WR_3	SE	WR_4	SE
Medpulser USA)	0.96	0.18	0.89	0.11	0.91	0.08
Home Made (Mexico)	1.00	0.10	0.72	0.08	0.79	0.04
GTH 1287 Jouan, (France).	0.65	0.31	0.33	0.08	0.50	0.13
Cliniporator IGEA (Italy) ESOPE	0.78	0.09	0.56	0.10	0.63	0.10
IQWave ChemoTech, (Sweden) D-EECT	1.00	0.35	0.60	0.16	0.70	0.15
Over All Average	0.80	0.06	0.57	0.08	0.66	0.07

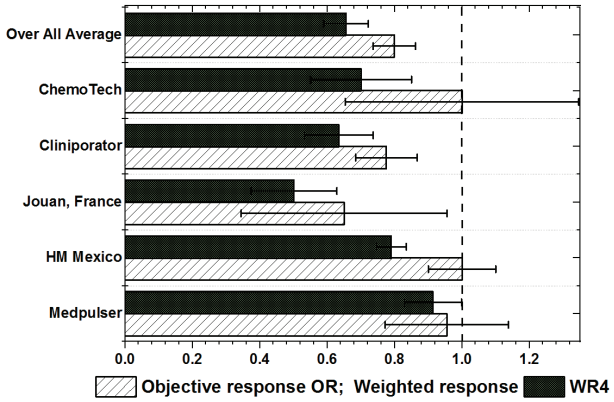

Figure 6-17
The objective responses and weighted response WR_4 of treating breast cancer with different devices

In treatment of Breast-cancer, the objective response was 100% for both the home-made Mexican device and the IQwave™ device closely followed by Medpulser™ 91%. The Jouan device and Cliniporator™ deliver objective responses of 65% and 78% respectively.

The Medpulser™ seems to deliver the best weighted response WR_4 91% for breast cancer followed by the home-made Mexican device with WR_4 79% and IQwave WR_4.70%. The Jouan device and ESOPE protocol with Cliniporator™ had the lowest weighted response with $WR_4$50% and 63% respectively. In those judgements, however, the sizes of tumours treated with the different devices were not considered. In the next paragraph, the treatment response of tumours of various sizes will be considered.

Figure 6-18 shows the objective response of squamous-cell carcinoma SCC of different tumour diameter treated with various types of devices. The Medpulser™ and IQwave™ seem both deliver 100% objective response, although the tumours treated with IQWave™ are twice as large. The electroporator-Jouan and Cliniporator™ seem both deliver an objective response of about 65%. Figure 6-19 shows the weighted response WR_4 of breast-cancer of different tumour diameter treated with various types of devices. At large tumour treatment (diam.≈5cm) the iQWave™-device delivered the best weighted response, WR_4≈60%, while the weighted response of Cliniporator™ was WR_4≈40% for tumours of corresponding size. By treatment of small tumours (diam.<3cm) with Cliniporator™, Jouan or Medpulser™, resulted in a weighted response WR_4 of about 45-50%

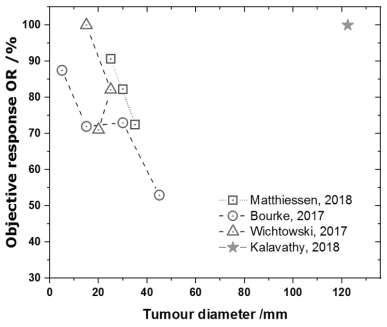

Figure 6-18
Objective response of Breast cancer of various sizes treated with different devices.
Cliniporator IGEA ESOPE:[177, 234, 236]
IQWave D-EECT Chemotech.[178]

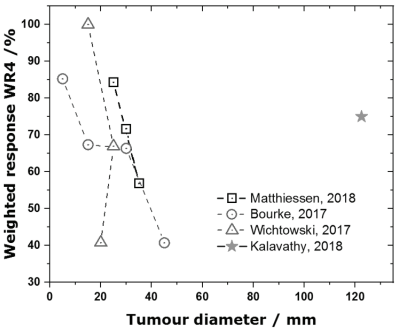

Figure 6-19
Weighted response WR4 of Breast cancer of various sizes treated with different devices
Cliniporator IGEA ESOPE:[177, 234, 236]
IQWave D-EECT Chemotech.[178]

6.1.5. BASAL CELL CARCINOMA (BCC)

The first clinical trial that performed for the treatment of cutaneous and subcutaneous tumours using electro-pulse enhanced chemotherapy to be analysed here involved two patients with 6 nodules of basal cell carcinoma. The treatment resulted in 12 % complete remissions and an 88% partial response.[179]

A second clinical study was initiated by the same researchers to determine whether an intra-lesional injection of the drug in combination with electric pulses could provide an improved result. A total of 54 Basal Cell Carcinoma primary tumours in 20 patients received a complete *EpEChT* treatment that resulted in a 96% complete response and 6 % partial response.[32]

Another clinical study of the effects of *EpEChT* on 20 patients with 54 primary BCC tumours resulted in 94% complete responses after a single treatment. No recurrences appeared within a mean of 18 months of observation.[238]

Five cancer centres performed independent clinical trials of electro-pulse enhanced chemotherapy in a total 32 basal cell carcinoma in 10 patients. Complete responses appeared in 75% and a partial response in 25%, which gives an objective response of 100%.[31]

A review of the clinical data for electro-pulse enhanced chemotherapy presented results for intravenous (i.v.) as well as intra-tumour administration of Bleomycin. Treatment of 2 BCC patients with i.v., resulted in 17% complete remissions and 83 % partial remissions, while treatment of 20 BCC patients with i.t. resulted in 84 % complete remissions and 6 % partial remissions.[194,239]

Further studies were initiated to confirm whether an intra-lesional injection of Bleomycin in combination with electric pulses could provide an improved result of electro-pulse enhanced chemotherapy treatment of BBC. In a group of totally 34 patients, of whom twenty were patients with 54 BCC nodules, there was 100% objective response, with 94% complete remissions and 6% partial remissions.[157]

From November 1998 through November 1999, 15 patients with 38 skin lesions participated in phase II prospective clinical trial, using intra-lesional Bleomycin plus electric pulses delivered 10min after Bleomycin injection, which lasted 0.1ms each at a field strength of 1300V/cm and a frequency of 1Hz. The study involved nine patients with basal cell carcinomas (BCC). After a follow-up of about nine months there was a 77.7% complete response and a 22.3% partial response.[158]

A pulse-sequence for electro-chemotherapy, consisting of 16 biphasic pulses of 25+25μs duration and intra-tumour Bleomycin, was used for treatment of clinical stage one basal cell carcinoma lesions in 45 patients. 80% of the patients achieved a complete response, and 20 % of the patients experienced recurrences.[196]

A case of a 75-year-old man with a pigmented nodule manifesting as the basal cell was treated with electro-pulse enhanced chemotherapy, resulting in a rapid clinical and histologic regression of the treated lesions.[240]

Electro-pulse enhanced chemotherapy successfully treated a man affected by ulcerated basal cell carcinoma. [241]

Between June 2009 and January 2011, three patients with Gorlin-Goltz syndrome were treated with electro-pulse enhanced chemotherapy using intravenous Bleomycin, according to the ESOPE guidelines. The patients had a total of 99 nodules of basal cell carcinomas on the face and trunk. The treated nodules showed 99% objective clinical response, with 87% complete response without recurrence during 10 to 28 months of follow-up,[242]

A recent review of the literature concludes that electro-pulse enhanced chemotherapy is an alternative to treating advanced basal cell carcinoma. However, standardized treatment schedules and randomized clinical trials were not considered.[174]

Summary of Basal Cell Carcinoma (BCC)

A summary of the results of treatment Basal Cell Carcinomas with Electro Enhanced Chemotherapy by using various devices reviewed in this section are given in Table 6-14a and 6-14b.

Table 6-14a
Results of treatment Basal Cell Carcinomas with Electro Enhanced Chemotherapy using different devices

Device Author[Reference]	No. Pat.	No. Tum.	fCR	fPR	fSD	fPD
Medpulser BTX USA						
Heller,1996[179]	2	6	0.13	0.88	0	0
Glass,1997[238]	10	32	0.75	0.25	0	0
Heller, 1998[32]	20	54	0.94	0.06	0	0
Heller,1999[194]	20	54	0.94	0.06	0	0
Heller,1999[194]	2	6	0.17	0.83	0	0
Heller,2000[157]	20	54	0.94	0.06	0	0
Home Made, Mexico						
Rodriguez-Cuevas,2001[158].	9	9	0.78	0.22	0	0

Table 6-14a continued

Chemipulser III, Bulgaria						
Peycheva,2004[196].	45	45	0.80	0.00	0	0.2
Jouan France						
Mir,1998[31]	10	31	0.75	0.25	0	0
ESOPE Cliniporator						
Fantini,2008[240]	1	3	1.00	0	0	0
Richetta,2011[241]	1		1.00	0	0	0
Kis,2012[242].	3	99	0.87	0.12	0.01	0
Over all Average	143	393	0.75	0.23	0,00	0.02
SD			0.30	0,31	0,00	0.06
SE			0.09	0.09	0,00	0.02

Table 6-14b

The objective (fOR) and weighted responses fWR_3 and fWR_4 of treatment Basal Cell Carcinomas with Electro Enhanced Chemotherapy using different devices.

Device Author[Reference]	No. Patients	No. Nodules	OR	WR_3	WR_4
Medpulser BTX USA					
Heller,1996[179]	2	6	1	0.42	0.56
Glass,1997[238]	10	32	1	0.83	0.88
Heller, 1998[32]	20	54	1	0.96	0.97
Heller,1999[194]	20	54	1	0.96	0.97
Heller,1999[194]	2	6	1	0.45	0.59
Heller,2000[157]	20	54	1	0.96	0.97
Home Made, Mexico					
Rodriguez-Cuevas,2001[158].	9	9	1	0.85	0.89
Chemipulser III, Bulgaria					
Peycheva,2004[196].	45	45	0.8	0.60	0.60
Jouan France					
Mir,1998[31]	10	31	1	0.83	0.88
ESOPE Cliniporator					
Fantini,2008[240]	1	3	1	1	1
Richetta,2011[241]	1		1	1	1
Kis,2012[242].	3	99	0.99	0.91	0.93
Sum					
Average	143	393	0.98	0.81	0.85
SD			0.06	0.21	0.17
SE			0.02	0.06	0.05

Table 6-15
Summary of Results of treatment Basal Cell Carcinomas with Electro Enhanced Chemotherapy by using different devices

Device	OR	SE	WR3	SE	WR4	SE
Medpulser BTX USA	1.00	0	0.76	0.11	0.82	0.08
HM Mexico	1.00		0.85		0.85	
Chemipulser III Bulgaria.	0.80		0.60		0.60	
Juan France	1.00		0.83		0.83	
ESOPE Cliniporator	1.00	0	0.79	0.03	0.79	0.02
Over all average	0.98	0.02	0.81	0.06	0,81	0,05

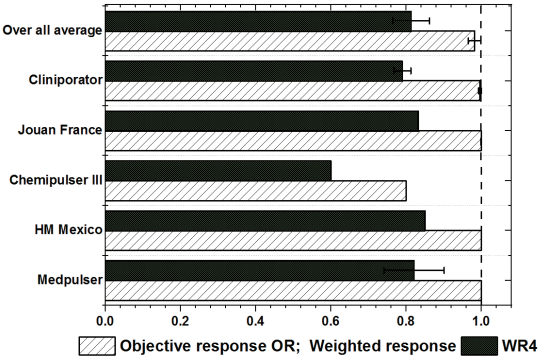

Figure 6-20
The objective responses and weighted response WR_4 of treating basal cell carcinoma by using different devices

6.1.6. KAPOSI´S SARCOMA

The first study of clinical applications of electro-pulse enhanced chemotherapy (*EpEChT*) involved one patient with four sites of Kaposi´s sarcoma. *EpEChT* treatment with intra tumoural administration of Bleomycin resulted in partial response of one of the sites treated while the other three showed progressive disease

A review of electroporation therapy of Kaposi's sarcoma mentioned *EpEChT* as an indication for treatment, and in the following years, several case reports were published.[243,244,160,245,246]

One included a two-centre prospective phase II clinical trial of electro-pulse enhanced chemotherapy which involved 23 patients with a total of

541 Kaposi`s sarcoma cutaneous nodules treated according to the ESOPE protocol. After a median follows up of 18 months, there were 65.2% complete remissions and 34.8% partial remissions, and at 24 months the survival rate was 74.4%.[247]

Clinical testing of newly developed electro-pulse enhanced chemotherapy equipment, with, heart QRS synchronizer, and the module for long-term recording of pulse parameters was carried out, involving 37 patients, some of whom had Kaposi´s Sarcoma. The publication, however, does not specify the outcome of the treatment of the Kaposi patients specifically.[248]

One prospective clinical study aimed at evaluating electro-pulse enhanced chemotherapy (*EpEChT*) with the intravenous administration of Bleomycin on 18 patients with 63 nodules of Kaposi's sarcoma. The objective response was 100% with 89% complete remissions and 11 % partial remissions.[249]

A single-centre clinical study was performed to evaluate *EpEChT* in the local treatment of stage I-II Kaposi's sarcoma. At 4 weeks after the first treatment, a 73.6 % complete response was achieved. The three patients who didn´t respond received a second treatment, and two of the patients got a third treatment until 100% complete remissions occured.[250,251]

Between 2008 and 2013 a prospective, multicentre, observational study of electro-pulse enhanced chemotherapy enrolled 40 patients with Kaposi`s sarcoma. The objective treatment results were 89%, with 44% complete remissions and 45% partial remissions, while 11 % experienced stable disease.[175]

A pilot study evaluating the effectiveness of electro-chemotherapy using reduced dosages of intravenous Bleomycin was carried out involving 40 patients with Kaposi´s sarcoma. However, in the report, there is no specific data about the outcome of those patients.[252]

A total of 27 patients with classic Kaposi´s sarcoma, were enrolled in a study aimed at evaluating the effect of *EpEChT* on HHV8 viral load. Tumour biopsies and blood samples were collected before and at six-month intervals for 12-48 months. The treatment resulted in 100% complete remissions that in the majority of cases was accompanied by clearance of the virus in the peripheral-blood mononuclear cells.[253]

Summary of Kaposi's sarcoma

A summary of the results of treatment Kaposi's sarcoma with Electro Enhanced Chemotherapy as reviewed in this section is given in Table 6-16.

Table 6-16a

Fractional Responses to Electro-pulse enhanced chemotherapy treatments of Kaposi's sarcoma using various devices.

Device Author[Reference]	No pat	No. Tum	fCR	fPR	fSD	fPD
BTX, Medpulser						
Heller, 1999[194]	1	1	0	1.00	0	0
Cliniporator, ESOPE						
Garbay, 2006[160]	1	8	1.00	0	0	0
Curatolo, 2008[245].	1	1	1.00	0	0	0
Gualdi, 2010[246]	1	1	1.00	0	0	0
Curatolo, 2012[247]	23	10-36	0.65	0.35	0	0
Latini, 2012[249]	18	63	0.89	0.11	0	0
Di Monta, 2014[251]	19	19	1.00	0	0	0
Campana, 2016[175]	40	40	0.44	0.45	0.11	0
Starita, 2017[253]	27	27	1.00	0	0	0
Sum	131	182	0.87	0.11	0.01	0
Average,						
SD			0.20	0.17	0.04	0
SE			0.07	0.06	0.01	0

Table 6-16b
The objective (fOR) and weighted responses fWR_3 and fWR_4 to Electro-pulse enhanced chemotherapy treatments of Kaposi's sarcoma using various devices.

Device Author[Reference]	No patients	No. Nodules	OR	WR_3	WR_4
BTX, Medpulser					
Heller, 1999[194]	1	1	1.00	0.33	0.50
Cliniporator, ESOPE					
Garbay, 2006[160]	1	8	1.00	1.00	1.00
Curatolo, 2008[245].	1	1	1.00	1.00	1.00
Gualdi, 2010[246]	1	1	1.00	1.00	1.00
Curatolo, 2012[247]	23	10-36	1.00	0.77	0.83
Latini, 2012[249]	18	63	1.00	0.93	0.94
Di Monta, 2014[251]	19	19	1.00	1.00	1.00
Campana, 2016[175]	40	40	0.89	0.59	0.69
Starita, 2017[253]	27	27	1.00	1.00	1.00
Sum/Average,	131	182	0.99	0.91	0.93
/SD			0.04	0.14	0.11
/SE			0.01	0.05	0.04

6.1.7. SOFT-TISSUE SARCOMA

Between October 2006 and March 2012, a two-clinic phase II trial of electro-pulse enhanced chemotherapy was conducted on 34 patients with locally advanced or metastatic soft-tissue sarcomas. The objective response outcome after up to four treatments was 76% with 32.4% complete remissions and 44.1% partial remissions, while 8.8% experienced stable disease and 14.7 progress disease.[254]

Between 2008 and 2013, ten centres performed a clinical electro-pulse enhanced chemotherapy study that enrolled 376 eligible patients of whom 10 were soft-tissue sarcomas. There is, however, no specific data about the outcome of treatment of soft-tissue sarcomas.[175]

Table 6-17 shows the summary of the various results of soft-tissue sarcoma treated with electro-pulse enhanced chemotherapy.

Table 6-17a
Treatment results of Soft Tissue Sarcoma with electro-chemotherapy.

Author[Reference]	No. patients	No. Nodules	fCR	fPR	fSD	fPD
Campana.2014[254]	34		0.324	0.441	0.088	0.147

Table 6-17b
The objective (fOR) and weighted responses fWR_3 and fWR_4 of Soft Tissue Sarcoma treatment with electro-chemotherapy.

Author[Reference]	No. patients	No. Nodules	fOR	fWR_3	fWR^4
Campana.2014[254]	34		0.76	0.32	0.42

6.1.8. SKELETON

The first test of electro-pulse enhanced chemotherapy as a treatment of spinal metastasis was performed in a 51-year-old female patient with a 30-mm metastatic spinal melanoma treated according to the ESOPE protocol with Bleomycin. During a 48-month follow-up, the target tumour showed complete remission, and the patient´s condition was noted as being in 'stable-disease'. Thus the treatment result of this single case of metastatic spinal melanoma with local control without adverse effects, indicates that electro-pulse enhanced chemotherapy has potential in the treatment of spinal metastasis.[255]

From July 2009 to July 2011, twenty-nine patients affected by painful bone metastases were treated with intravenous Bleomycin electro-pulse enhanced chemotherapy according to the ESOPE protocol. At 3 months after the treatment, 20 patients showed 5% a partial response, 85% stable disease, and 10% progression of disease. After 7-month follow-up of 18 patients, showed 61% a partial response, and 40% progression of the disease. After 12-months' follow-up of 11 patients, showed 64% a partial response and 36% progression of disease.[256]

Table 6-18 displays the treatment results of spinal and bone metastases with electro-pulse enhanced chemotherapy.

Table 6-18a

Treatment results of spinal and bone metastases with electro-pulse enhanced chemotherapy.

Author[Reference]	No. Patients	Follow up month	fCR	fPR	fSD	fPD
Gasbarrini,2015[255]	1	48	1.00	0	0	0
Bianchi,2016-a[256]	20	3	0	0.05	0.85	0.10
Bianchi,2016-c[256]	18	7	0	0.61	0	0.39
Bianchi,2016-c[256]	11	12	0	0.64	0	0.36
Sum	50	18				
Average			0.25	0.32	0.21	0.21
/SD		18	0.43	0.30	0.37	0.17

Table 6-18b

The objective (fOR) and weighted responses fWR_3 and fWR_4 of spinal and bone metastasis's treatment with electro-pulse enhanced chemotherapy.

Author[Reference]	No. Patients	Follow up month	OR	WR_3	WR_4
Gasbarrini,2015[255]	1	48	1.00	1.00	1
Bianchi,2016-a[256]	20	3	0.05	-0.08	0.14
Bianchi,2016-b[256]	18	7	0.61	-0.19	0.09
Bianchi,2016-c[256]	11	12	0.64	-0.15	0.04
Sum	50	18			
Average			0.57	0.14	0.25
/SD		18	0.34	0.50	0.25

6.1.9 LIVER

In a clinical phase I/II study of electro-pulse enhanced chemotherapy with Bleomycin during open surgery, by insertion of long needle electrodes in 29 colorectal liver metastases on 19 patients no, serious adverse events occurred. Radiological evaluation the treated metastases showed 85% complete responses and 15% partial responses.[257]

Histopathological findings in the colorectal liver metastases after electro-chemotherapy showed induced coagulation necrosis in the treated area encompassing both tumour and a narrow band of healthy tissue. Further evidence of disruption of vessels less than 5 mm in diameter and preservation of the larger vessels by electro-chemotherapy occurred. No viable tumour cells are found around the larger vessels in the treatment

volume. Around 9 weeks after electro-chemotherapy, regenerative changes occur.[258]

In a feasibility study of the treatment of portal-vein tumour thrombosis at the hepatic hilum in patients with hepatocellular carcinoma in cirrhosis, electro-pulse enhanced chemotherapy seems effective and safe for curative treatment.[259] The electro-pulse enhanced chemotherapy resulted in complete remissions in all treated nodules. Although 3 patients died within 1-14 months due to rupture of oesophageal varices or liver failure. The high risk of the occurrence of this complication might indicate that patients with gastric or oesophageal varices, it should be excluded from treatment with electro-pulse enhanced chemotherapy of the liver.[259]

Electro-chemotherapy with Bleomycin combined with open liver resection and performed with linear or hexagonal needle electrodes to 9 colorectal liver metastases in five patients according to the ESOPE protocol with 1-3 applications per nodule at 730V/cm resulted in 55.5% complete response, and 45.5% stable disease with no intraoperative complications. All five patients reached a 6 months' overall survival, and 4 out of 5 patients had 6 months' progression-free survival.[260]

6.1.10 OTHER DIAGNOSIS

Sweat gland carcinoma, *Eccrine poro-carcinoma*

A case of the patient with *Eccrine poro*-carcinoma, a rare type of skin cancer that arises from the intra-epidermal ductal portion of the eccrine sweat gland on the left arm with axillary nodal involvement, was treated by complete lymph node dissection and electro-pulse enhanced chemotherapy. After a follow-up of five months, complete response observed on a clinically macroscopic basis with no signs of axillary relapse or systemic disease.[261]

Gastric cancer

A case of a 49-year-old man with advanced gastric cancer developed skin metastases around an ileostomy site was treated with electro-pulse enhanced chemotherapy according to the ESOPE protocol with i.v. Bleomycin. At one-month follow-up, partial tumour regression observed.[262]

Parotid gland

In a case study, one metastasis in the left parotid gland and another metastasis in the neck was treated with electro-pulse enhanced chemotherapy performed using intravenous administration of Bleomycin and long single needle electrodes. The neck nodule got complete remission

while the parotid nodule got partial remission. Six weeks after electro-chemotherapy, fine-needle aspiration biopsy of the treated area revealed necrosis and inflammatory cells, without any viable tumour cells in the specimens from both of the treated lesions.[263]

Endometrial cancer

The first case of cutaneous metastases from endometrial cancer treated with electro-pulse enhanced chemotherapy was a patient with multi-metastatic endometrial cancer. The patient received palliative systemic therapy, and electro-pulse enhanced chemotherapy according to the ESOPE guidelines. The treatment resulted in partial recovery, with necrosis at the treatment site and interruption of lesion bleeding.[264]

Prostate cancer

A case report describes the successful electro-pulse enhanced chemotherapy treatment of a patient with prostate cancer with infiltration of the urethral sphincter. Four electrodes were inserted percutaneously through the perineum, each in pairs ventrally and dorsally of the sphincter externus arranged in a square. Electro-pulse enhanced chemotherapy performed according to the ESOPE protocol. Toxicity of the treatment was low, with only mild adverse events. At 6 months after the treatment, the patient was in complete remission with mild incontinence and mild erectile dysfunction.[265]

Pancreatic adenocarcinoma

During the period 2011 - 2014 a clinical phase I/II study aimed to evaluate the effect of Electro-pulse enhanced chemotherapy in the treatment of locally advanced pancreatic adenocarcinoma enrolled 6 females and 5 male patients. Preliminary results show that for 5 patients no significant reduction of maximal tumour diameter appeared in CT and MR imaging procedures. However, after about one month from *EpEChT*, all patients showed a significant reduction of functional parameters value associated with a partial response.[266]

Eleven patients with locally advanced tumours of the head or the body of the pancreas managed treatment with electro-pulse enhanced chemotherapy compared to pre-operative status the patients with locally advanced pancreatic tumours reported pain reduction immediately after the electro-pulse enhanced chemotherapy treatment, and better quality of life without severe side effects or significant complication.[267]

In a clinical phase I/II study of electro-pulse enhanced chemotherapy functional MRI visualized significant reduction of advanced pancreatic

adenocarcinoma in the treated target area of patients at the National Cancer Institute, "G. Pascale Foundation" of Naples. During post-operative PET-examination, it was also indicated that the ^{18}FDG uptake was lower in respect to pre-operative evaluations.[268]

Lung Cancer

Despite the high proportion of complete remissions of various types of cancer reported in the ESOPE study, there is no clinical study of electro-pulse enhance chemotherapy treatment of lung cancer reported.[162] But considering current attempts of thermal ablation for treatment of lung tumours indicates that electro-pulse enhanced chemotherapy might represent a significant breakthrough in the treatment of lung cancer.[269]

Summary of various types of tumours

Table 6-19 shows a summary of the results of treatment various other types of tumours with electro-pulse enhanced chemotherapy reviewed in this section.

Table 6-19a
Results of electro pulse enhanced chemotherapy of various other types of tumours than above

Author[Reference]	Tumour type	Follow up month	fCR	fPR	fSD	fPD
Marone,2011[261]	Sweat gland	5	1.00	0	0	0
Campana,2013[262]	Gastric cancer	1	0	1.00	0	0
Groselj,2015[263]	Parotid gland	1.5	1.00	0	0	0
Schiavii,2017[264]	Endometrial ca.	4	0	1.00	0	0
Klein,2017[265]	Prostate ca.	6	1.00	0	0	0
Granata,2014[266]	Pancreatic ad.ca.	1	0	1.00	0	0
Tafuto,2015[267]	Pancreatic ad.ca.	1	0	1.00	0	0
Bimonte,2016[268]	Pancreatic ad.ca.	3	0	1.00	0	0
Jahangeer,2013[269]	Lung cancer	-	-	-	-	-
Average		21	0.38	0.63	0	0

Table 6-19b
The objective (fOR) and weighted responses fWR_3 and fWR_4 to electro pulse enhanced chemotherapy of various other types of tumours than above.

Author[Reference]	Tumour type	Follow up month	OR	WR_3	WR_4
Marone,2011[261]	Sweat gland	5	1.00	1.00	1.00
Campana,2013[262]	Gastric cancer	1	1.00	0.33	0.50
Groselj,2015[263]	Parotid gland	1.5	1.00	1.00	1.00
Schiavii,2017[264]	Endometrial cancer	4	1.00	0.33	0.50
Klein,2017[265]	Prostate cancer	6	1.00	1.00	1.00
Granata,2014[266]	Pancreatic ad.ca.	1	1.00	0.33	0.50
Tafuto,2015[267]	Pancreatic ad.ca	1	1.00	0.33	0.50
Bimonte,2016[268]	Pancreatic ad.c.a	3	1.00	0.33	0.50
Jahangeer,2013[269]	Lung cancer	-	-	-	-
Average		21	1.00	0.58	0.61
SD		2		0.35	0.33
SE		0.8		0.13	0.11

6.1.11 SUMMARY OF ALL *EpEChT* CLINICAL STUDIES WITH BLEOMYCIN

Table 6-20 gives a summary of the results all electro-pulse enhanced chemotherapy clinical studies with Bleomycin reviewed in this chapter.

Table 6-20a
Summary of all results of clinical studies with Bleomycin electro-enhanced chemotherapy of various other types of tumours.

Type of Ca.	fCR	fPR	fSD	fPD
Malignant Melanoma	0.49±0.08	0.30±0.06	0.16±0.3	0.04±0.02
Squamous cell carcinoma	0.38±0.05	0.35±0.05	0.15±0.03	0.12±0.04
Breast Cancer	0.51±0.07	0.29±0.06	0.16±0.06	0.04±0.02
Basal Cell Carcinoma	0.75±0.09	0.23±0.09	0	0.02±0.02
Kaposi´s sarcoma	0.87±0.07	0.11±0.01	0.01±0.04	0.00±0.00

Table 6-20a continued

Soft Tissue *Sarco-sarcoma*	0.32	0.44	0.09	0.15
Skeleton metastases	0.25±0.20	0.32±0.15	0.21±0.19	0.21±0.09
Liver metastases	0.57±0.40	0.14±0.24	0.11±0.19	0.18±0.26
Other tumours	0.38±0.20	0.63±0.20	0	0
Average±SD	0.50±0.20	0.31±0.16	0.10±0.08	0.08±0.08
Average±SE	0.50±0.07	0.32±0.05	0.10±0.03	0.08±0.03

Table 6-20b

The objective (fOR) and weighted responses fWR_3 and fWR_4 to all results of clinical studies with Bleomycin electro-enhanced chemotherapy of various other types of tumours.

Type of Ca.	No. Patients	No. Nodules	fOR	fWR_4
Malignant Melanoma	376	1799	0.80±0.5	0.55±0.07
Squamous cell carcinoma	562	669	0.73±0.04	0.48±0.06
Breast Cancer	433	1455	0.80±0.06	0.66±0.07
Basal Cell Carcinoma	143	393	0.98±0.02	0.85±0.05
Kaposi´s sarcoma	131	1182	0.99±0.01	0.93±0.04
Soft Tissue *Sarco-sarcoma*	34	34	0.76	0.42
Skeleton metastases	50	50	0.57±0.16	0.25±0.12
Liver metastases	17	9	0.71±0.29	0.49±0.51
Other tumours	9	9	1.00	0.61±0.11
Sum	1755	5600		
Average±SD			0.82±0.15	0.58±0.21
Average±SE			0.82±0.05	0.58±0.07

Kaposi´s sarcoma, and Basal Cell Carcinoma are the types of tumours most successfully treated with Bleomycin electro-pulse enhanced chemotherapy, with objective responses close to 100% and weighted response WR_4 close to 90%.

The second most successful treatment of malignant melanoma and breast cancer achieved an objective response of about 80% with weighted response 60% due to about 4% of progressive disease. Thus more effort should be focused to improve the treatment of malignant melanoma and breast cancer.

The treatment results for liver metastases is also promising with an objective response of treatment about 70%. The weighted response is, however, only about 40% due to a high percentage of about 20% progressive

disease. Thus more effort should be focused to improve the treatment of liver metastases.

The overall result of electro-pulse enhanced chemotherapy is auspicious with an average objective response of 82% and an average weighted response of about 60%. The future development should be focused to enhance the response of complete remissions and reduce the fraction of progressive disease.

6.2 CLINICAL STUDIES OF *EpEChT* WITH CIS-PLATIN

6.2.1 INTRODUCTION

The first study performed to study electro-pulse enhanced chemotherapy with Cisplatin was cutaneous tumour nodules in four patients with 19 nodules of malignant melanoma, squamous-cell carcinoma, and basal cell carcinoma. Cisplatin was administered intra-tumoural at doses depending on the size of the tumour nodule according to Figure 6-21.

The variation of the administrated intra-tumour dose of Cisplatin(Pt) with the size of the tumour nodule is represented by the values given in *Table 2* of the publication by Sersa.1998[270] follows the equation:

$$\text{Cis-Pt dose (mg)} = 0.2 + 0.01 \times \text{TV(mm}^3\text{)}$$
$$\text{in the interval } 1 < \text{TV(mm}^3\text{)} < 1000$$

Electric pulses were delivered one per second, through two parallel stainless steel electrodes, with a constant amplitude of 1300V per cm of electrode distance (910V/cm) and 0.1ms in duration. At a four-weeks follow-up, a complete response (CR) occurred in all 19 electro-chemotherapy treated nodules, and they all remained without progression up to 7-11 months.[270]

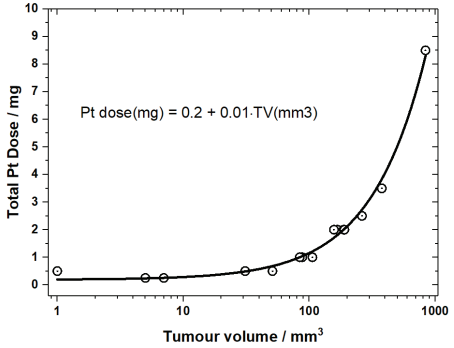

Figure 6-21
The administrated intra-tumour dose of cisplatin(Pt) extracted of the values given in *Table 2* of the publication by Sersa 1998.[270]

A two year-long prospective non-randomised study with four cancer centres enrolled 14 patients with breast cancer to evaluate and confirm the efficacy and safety of electro-pulse enhanced chemotherapy with Bleomycin or Cisplatin. The treatment of recurrent breast cancer was performed using an intravenous or intra-tumoural drug, followed by application of electric pulses generated by a Cliniporator™ using plate or needle electrodes. In the 58 breast cancer nodules treated with electro-pulse enhanced chemotherapy, a complete response appeared in 84 % of the nodules, partial response in 7 %, and stable disease in 10%, regardless use of drug or route of its administration.[162]

Intra-tumour administration of Cisplatin is recommended by *European Standard Operating Procedures of Electro chemotherapy* (ESOPE) to depend on tumour volume according to the follow rules:

Cisplatin. dissolved at a concentration of 2 mg/ml given intratumoural only.

- For tumour > 1 cm^3 0.5 mg/cm^3 CisPt i.t Dose = 0.5 ×TV [mg]
- For tumour 1 - 0.5 cm^3 1 mg/cm^3 CisPt i.t Dose = 1 ×TV [mg]
- For tumour < 0.5 cm^3 2 mg/cm^3 CisPt i.t Dose = 2 ×TV [mg]

Electric pulses were applied to the tumour nodules as soon as possible within 2min after the intra-tumoural injection of Cisplatin.[165]

6.2.2 MALIGNANT MELANOMA

A Phase II clinical study was performed to evaluate the antitumor effectiveness of electro-pulse enhanced chemotherapy using intra-tumour Cisplatin administration on cutaneous tumour nodules in 10 malignant melanoma patients with 133 tumour nodules of different sizes.[271]

Cisplatin was administered intra-tumorally at doses depending on the size of the tumour nodule according to the equation:

$$\text{Pt-dose (mg)} = 0.01 \times \text{TV}(mm^3)$$

up to 200 mm^3, then 2 mg for all volumes above 200 mm^3.

Eighty-two tumour nodules treated with cisplatin electro-chemotherapy; 27 tumour nodules were treated with cisplatin only; 2 tumour nodules were treated. with electric pulses only; and 22 tumour nodules were untreated. After four weeks the fraction of complete response in the electro-pulse enhanced chemotherapy group was 68% and partial response was 10%. which corresponds to 78 % objective responses, and 15% experienced stable disease and 5% progressive disease, while in the objective response in the

cisplatin group was 38%, and in the electric pulses only there was no response.[271] In a previous study of 4 patients with 19 nodules the complete response was 100 %.[270] Thus electro-pulse enhanced chemotherapy with cisplatin was judged to be a highly effective approach for treatment of cutaneous malignant melanoma nodules.[271.272]

A case with a large anorectal malignant melanoma was preoperatively treated by electro-pulse enhanced chemotherapy with Cisplatin to reduce the tumour size, to enable Sphincter-saving local excision. After surgery, radiation-brachytherapy was delivered to the excision site, and at 14 months' follow-up there, was no sign of recurrence.[273]

Another case of melanoma on the calf with multiple cutaneous and subcutaneous metastases treated by electro-pulse enhanced chemotherapy with Cisplatin showed a long-lasting complete response.[167]

Table 6-21a

The reported responses of cis-Pt *EpEChT* of Malignant Melanoma.

Author[Reference]	No. Patients	No. nodules	*f*CR	*f*PR	*f*SD	*f*PD
Sersa,1998[270]	4	19	1	0	0	0
Sersa,2000[271]	10	82	0.68	0.10	0.15	0.05
Sersa,2003[274]	3	10	0.50	0.20	0.30	0
Snoj,2005[273]	1	1	1	0	0	0
Snoj,2006[167]	1	16	0.81	0	0	0.23
Sum	19	128				
Average			0.80	0.06	0.09	0.06
SD			0.21	0.09	0.13	0.10
SE			0.10	0.04	0.06	0.04

Table 6-21b

Objective (fOR) and weighted responses fWR_3 and fWR_4 to Cis-Pt *EpEChT* of Malignant Melanoma.

Author[Reference]	No. Patients	No. nodules	*f*OR	fWR_3	fWR_4
Sersa,1998[270]	4	19	1	1	1
Sersa,2000[271]	10	82	0.78	0.70	0.72
Sersa,2003[274]	3	10	0.70	0.57	0.68
Snoj,2005[273]	1	1	1	1	1
Snoj,2006[167]	1	16	0.81	0.58	0.58
Sum/Average	19	128	0.86	0.77	0.79
/SD			0.14	0.22	0.19
/SE			0.06	0.10	0.09

6.2.3 BREAST CANCER BC

In a study treating two patients with 12 cutaneous breast cancer lesions of by electro-pulse enhance chemo-therapy with Cisplatin, a complete response appeared in 33% and partial response in 67 %, while no complete responses occurred with i.t. application of Cisplatin alone.[237]

Table 6-22a
Reported responses of Cis-Pt *EpEChT* treatment of Breast Cancer

Author[Reference]	No. Patients	No. nodules	*f*CR	*f*PR	*f*SD	*f*PD
Rebersek,2004[237]	2	12	0.33	0.67	0	0

Table 6-22b
Objective (fOR) and weighted responses fWR_3 and fWR_4 to Cis-Pt *EpEChT* treatment of Brest Cancer

Author[Reference]	No. Patients	No. nodules	*f*OR	fWR_3	fWR_4
Rebersek,2004[237]	2	12	1	0.55	0.67

6.2.4 SUMMARY OF ALL *EpEChT* CLINICAL STUDIES WITH CIS-PLATIN

Table 6-23a
The reported responses of cis-Pt *EpEChT* treatment of various types of tumours

Diagnosis	No. Pat.	No. Tum.	*f*CR	*f*PR	*f*SD	*f*PD
Malignant Melanoma	19	128	0.80	0.06	0.09	0.06
Squamous Cell Ca	1	2	1	0	0	0
Basal Cell Ca.	1	9	1	0	0	0
Breast cancer	2	12	0.33	0.67	0	0
Sum	23	151				
Average			**0.78**	**0.18**	**0.02**	**0.01**
SD			0.32	0.33	0.05	0.03
SE			0.22	0.23	0.03	0.02

Table 6-23b
Objective (fOR) and weighted responses fWR_3 and fWR_4 to cis-Pt *EpEChT* treatment of various types of tumours

Diagnosis	No. Patients	No. nodules	*f*OR	fWR_3	fWR_4
Malignant Melanoma	19	128	0.86	0.77	0.79
Squamous Cell Ca	1	2	1	1	1
Basal Cell Ca.	1	9	1	1	1
Breast cancer	2	12	1	0.55	0.67
Sum	23	151			
Average			0.96	0.83	0.86
SD			0.07	0.22	0.17
SE			0.05	0.15	0.08

Table 6-24a
The response ratios to Cis-Pt *EpEChT* / Bleomycin *EpEChT* treatments of various types of tumours

Diagnosis	Pt/Bleo CR	Pt/Bleo. PR	Pt/Bleo. SD	Pt/Bleo. PD
Malignant Melanoma	1.76	0.18	0.54	1.29
Squamous Cell Ca.	2.37	0	0	0
Basal Cell Ca.	1.32	0	0	0
Breast cancer	0.63	2.53	0	0
Average	1.52	0.68	0.14	0.32

Table 6-24b
Objective OR and weighted WR_3 and WR_4 response-ratios to Cis-Pt *EpEChT* / Bleomycin *EpEChT* treatments of various types of tumours

Diagnosis	Pt/Bleo. OR	Pt/Bleo. WR_3	Pt/Bleo. WR_4
Malignant Melanoma	1.10	1.48	1.44
Squamous Cell Ca	1.34	2.44	1.82
Basal Cell Carcinoma	1.02	1.23	1.18
Breast cancer	1.24	0.97	1.01
Average	1.18	1.53	1.36

In Table 6-24 are given the ratios of the average responses for treatment of various types of tumours with Cis-Pt *EpEChT* (Table 6-23) and corresponding tumours with Bleomycin *EpEChT* (Table 6-20). The corresponding ratios for objective and the WR_4 weighted responses are displayed in Figure 6-22. Although the number of cases are sparse, squamous-cell carcinoma seems to respond much better, and malignant melanoma seems to respond slightly better with Cis-Pt *EpEChT* than with Bleomycin *EpEChT*. For Basal cell carcinoma and breast cancer there seems to be no difference.

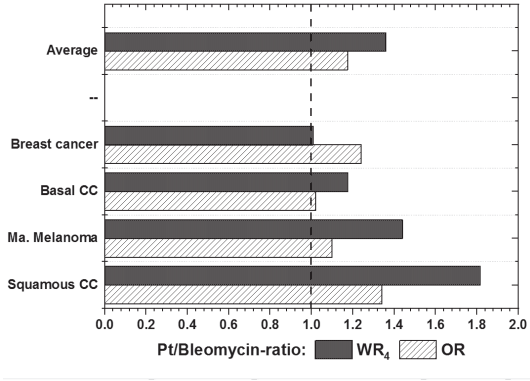

Figure 6-22
Response ratios of Cis-Pt *EpEChT* versus Bleomycin *EpEChT* objective OR and weighted WR_4 responses for treatment of various types of tumours.

According to the ESOPE recommendation, Cisplatin should only be administered at a concentration of 2 mg/ml, although the total administered dose varies with the tumour volume and the number of therapy sessions as shown in Figure 6-23.

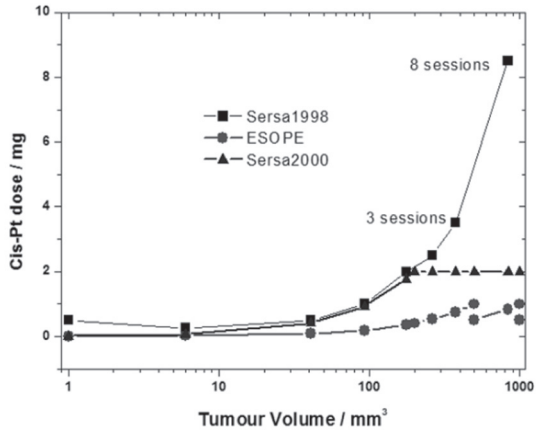

Figure 6-23
Variation of total administered dose of Cis-Pt with the tumour volume and the number therapy sessions as used in clinical studies.[270,271,272] and recommended by ESOPE.[165]

6.3 CLINICAL STUDIES OF *EpEChT* WITH OTHER PHARMACEUTICALS

6.3.1 CLINICAL STUDIES OF *EpEChT* WITH INTERLEUKINS

Treatment regimens combining electro-pulse enhanced cancer therapy (EpECT) to biological response modifiers (interleukin-2, interferon) and immunotherapeutic compounds is presently intensively explored in animal and human cancer models. The hope is that immunotherapy combined to EpECT could broaden the therapeutic indications of EpECT to deep-seated and spread disease.

In an attempt to analyse anti-tumour immune response in patients treated with IL-2 based immunotherapy systemic anti-tumour cytotoxic T lymphocyte (CTL) responses was studied against known tumour antigens in melanoma patients over the course of IL-2 based immunotherapy combined with electrochemotherapy.[275,276,277] Surprisingly, they found that anti-tumour CTL responses significantly declined upon initiation of therapy, but reappeared when pausing IL-2 administration. Molecular analyses of the clono-typic composition of responding T cells demonstrated that new clones emerged during the treatment, and that tumour-specific T cells left the peripheral blood and could subsequently localize at the tumour site. This underlines the importance to gain detailed information on the interactions between cancer cells and cells of the immune system.

The US company *OncoSec Medical* has three ongoing Phase 2 trials in treating metastatic melanoma, which support that electro-pulse treatment combined with the agent IL-12 (*ImmunoPulse)* to 47 melanoma patients showed a systematic immune response with tumour shrinkage.

Merkel-cell carcinoma (MCC) is an orphan disease with no Food and Drug Administration-approved treatments so far. However, *OncoSec Medical* is conducting trials in treating MCC with '*ImmunoPulse*,' in which they record an uptake of IL-12 of at least 100-fold, and some as high as the 1,000-fold increase in patients treated.[278]

6.3.2 CLINICAL STUDIES OF *EpE*-DELIVERY OF DNA VACCINES

In a review. the use of electro-pulse enhanced DNA delivery to enhance the immune responses, the cancer antigens, and the escape mechanism(s) used by tumour cells are discussed, with a focus on the progress of clinical trials using cancer DNA vaccines.[279]

In phase I/II trial a DNA vaccine, SCIB1, incorporating two CD8 and two CD4 epitopes from TRP-2/gp100 has evaluated in patients with metastatic melanoma. The patient tolerated the delivery of the DNA vaccine well, and the vaccine stimulated potent T cell responses in melanoma patients.[280]

6.3.3 *EpEChT* WITH PD-1. CTLA-4 IMMUNE CHECKPOINT BLOCKADE

Anti-PD-1 agents and anti-CTLA-4 agents have been shown to improve progression-free survival and objective response rate as monotherapy in patients with advanced melanoma. However, they are not adequate for all cases and associated with significant toxicity.[281]

Table 6-25 displays the results of a retrospective study ipilimumab in combination with electro-pulse enhanced chemo-therapy *EpEChT* which involved fifteen patients with previously treated metastatic melanoma. The patients included in the analysis received ipilimumab 3mg/kg every three weeks for 4 cycles. After the first Ipilimumab infusion, they underwent *EpEChT* with Bleomycin 15mg/m^2.[282]

Table 6-25a

Treatment response to melanoma patients with a combination of Bleomycin *EpEChT* and the anti-CTLA-4 agent Ipilimumab.

Diagnosis	No. Patients	Response	*f*CR	*f*PR	*f*SD	*f*PD
Malignant Melanoma	15	Local	0.27	0.40	0.33	0
Malignant Melanoma	9	Systemic	0	0.30	0.26	0

Table 6-25b

The objective OR and weighted WR_3 and WR_4 treatment response to melanoma patients with a combination of Bleomycin *EpEChT* and the anti-CTLA-4 agent Ipilimumab.

Diagnosis	No. Patients	Response	*f*OR	*f*WR_3	*f*WR_4
Malignant Melanoma	15	Local	0.67	0.40	0.55
Malignant Melanoma	9	Systemic	0.56	0.30	0.22

Another clinical evaluation of the use of electro-pulse enhanced chemotherapy (EEChT) combined with CTLA-4 or PD-1 inhibitors involved 33 patients with unresectable or metastatic melanoma. The patients received combination of *EpEChT* and immune checkpoint blockade for distant or cutaneous metastases within 4 weeks. Table 6-26 shows a

summary of the treatment results. The anti-CTLA-4 agent Ipilimumab combined with *EpEChT* showed a high systemic response rate. The local response, however. was lower than reported for electro-pulse enhanced chemotherapy *EpEChT* only.[283]

A case history reports a complete response after electro enhanced chemotherapy on a progressive mass of metastatic melanoma (MM), while anti-PD-1 treatment with Nivolumab, with no evidence of disease at 4 years since previous local recurrence, combined with anti-PD-1 treatment exhibited induced durable complete response in the heavily pre-treated metastatic melanoma patient.[284]

Table 6.26
The response of combination of Bleomycin EpECT treatment with anti PD-1 and anti-CTLA-4 agents.

No. Pat.	Agent	Local response %	Systemic response %	Time to progression month	Toxicity Event %
28	Anti CTLA-4	66.7	19.2	2	25
5	Anti PD-1	66.7	40	5	

6.3.4 ELECTRO ENHANCED THERAPEUTIC EFFICIENCY OF CALCIUM

A double-blinded randomized study was performed to compare calcium electroporation to electro-chemotherapy concerning the response measured 6 months after treatment.[285] The study protocol included seven patients with a total of 47 cutaneous metastases from breast cancer and malignant melanoma with a total of 37 metastases randomized for treatment. The patients received intratumoural administered calcium (≈50μmol) or Bleomycin (≈200IU) followed by application of electric pulses at average electro- doses of respectively 700 and 400J/g respective to the tumour site. Table 6-27 shows the treatment results, which do not indicate any significant difference between the two treatments.

Table 6-27a
The fractional responses of Calcium and Bleomycin *EpEChT* treatment

Drug	Dose	CR	PR	SD	PD
Bleomycin (1000IU)	i.t 0.2 ml	0.68	0.15	0	0.15
$CaCl_2$ (220mM)	i.t 0.2 ml	0.66	0.05	0.16	0.11

Table 6-27b
Objective OR and weighted WR_3 and WR_4 treatment response to Calcium and Bleomycin *EpEChT* treatments

Drug	Dose	OR	WR_3	WR_4
Bleomycin (1000IU)	i.t 0.2 ml	0.83	0.58	0.61
$CaCl_2$ (220mM)	i.t 0.2 ml	0.71	0.57	0.62

Since the patients who received calcium treatment on average received larger deposited electric energy, their responses are not entirely comparable with the Bleomycin treatment. This study, however, clearly show the benefit of using Ca in electro enhanced cancer-therapy, like in electro enhanced weed control (see 4.6.1).

CHAPTER VII

SAFETY OF ELECTRO ENHANCED CHEMOTHERAPY

7.1 BIOLOGICAL EFFECTS OF ELECTRIC SHOCK

7.1.1 TOLERANCE OF ELECTRICAL SENSATION INDUCED BY ELECTRIC FIELDS

Exposure of patients to intense electric pulses is known to affect cell membranes in the exposed tissues. At exposure to a strong electric current and high absorbed electric energy, denaturation of macromolecules and complex biochemical reactions occur.

The field-induced transmembrane potential can produce electro-conformational changes of ion channels and ion pumps in the cell membranes. If the potential exceeds the dielectric strength of the cell membrane (approximately 0.5 V for a pulse width of a few ms), electro conformational changes of membrane lipid bilayers create electroporation. These events cause the electric current to pass through the porated cell membrane and affect the cytoplasmic contents of the cells. Besides electroporation of cell membranes and denaturation, cytoplasmic macromolecule brings about many complex biochemical reactions such as oxidation of proteins and lipids. The combined effects of all these events may damage the cells beyond possible repair. Lipid peroxidation and the subsequent loss of the energy-transducing ability of the cells may occur even at moderate temperatures between 40°C and 45°C.[286]

A clinical study enrolled 20 patients in order to evaluate the safety and human tolerance of electrical sensation induced by electric fields with non-invasive electrodes. The patients were subjected during 3ms to single pulses of 50–80 V amplitude escalating in 10-V increments. Table 7-1 shows the patients' rating of the pulse sensation on a scale of 0, no pain, to 10, excruciating pain.

The summary of the results displayed in Figure 7-1 indicates that the tolerability starts to decrease above 80 V. Thus, exposure to higher amplitudes requires local anaesthesia to be applied.[287,288]

In addition to the sensation of pain caused by high voltage pulse delivery, muscle contractions present other unpleasant sensations for patients undergoing electro-chemotherapy. Since each single pulse that was delivered was associated with muscle contractions, studies have been conducted in an attempt to reduce the number of unpleasant sensations by increasing the frequency of electric pulses over the frequency of tetanic contraction. Measurements of muscle torque in rats showed that exposure to electric fields of pulse frequencies above the frequency of sustained muscle contraction (> 100 Hz) reduces the number of individual contractions to a single muscle contraction.[289]

Summary of the results displayed in Figure 7-1 indicates that the tolerability starts to decrease above 80 V. Thus, exposure to higher amplitudes requires local anaesthesia to be applied.[287,288]

Regardless of the pulse amplitude, with increasing pulse frequency, muscle torque increases up to the frequency of 100 or 200 Hz and then decreases to a value similar to that after application of a 1 Hz pulse train. Electro-chemotherapy in vivo with higher repetition frequencies inhibits tumour growth and is efficient at all pulse frequencies examined (1 Hz - 5 kHz). These results suggest that there is considerable potential for clinical use of high-frequency pulses in electro-chemotherapy.[289]

According to their results, the muscle torque grows with an increase of the repetition frequency, and at the frequency 5 kHz the muscle torque was two times higher than at 1 Hz. They also found that electro-pulse-enhanced chemotherapy of a tumour model in mice at 1 Hz or 5 kHz has about the same efficiency (at 32% versus 22% complete remissions respectively). Thus, application of a 5 kHz pulse train with 8 pulses instead of 8 individual pulses at 1 Hz has the advantage of only one sensation instead of eight with the same outcome of the treatment.[289]

Table 7-1
Score table of the electric pulse sensation

Score	Sensation
0	No pain
1-2	Mild
2-4	Discomforting
5	BETWEEN
6-7	Distressing
8-9	Horrible
10	Excruciating

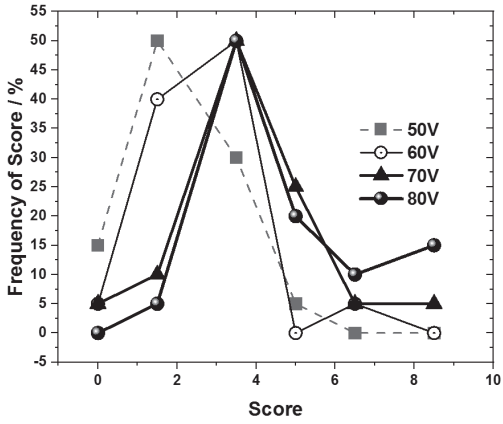

Figure 7-1
Frequency of score for pain sensation at exposure to cutaneous electric fields during 3 ms at various amplitudes.

7.1.2 TOLERANCE OF PAIN AND MUSCLE CONTRACTIONS INDUCED BY ELECTRIC FIELDS

A clinical study was designed to analyse the safety of electro-pulsed-enhanced chemotherapy, and involved 61 patients. The use of low doses of the chemotherapeutic preparations limits adverse reactions related to treatment with Bleomycin or Cisplatin.

For the relief of symptoms associated with the application of electrical pulses, 60% of the patients used local anaesthesia, and 40% used general anaesthesia. In patients with local anaesthesia, the median level of pain immediately after treatment was 35 units in a range 0-100 of visual assessment. Two days after treatment, the median pain level had dropped to 20 units. Patients treated with general anaesthesia reported significantly lower pain than those treated with local anaesthesia. The pain was assessed as acceptable and was limited to the area of the treated tumour. In connection with the application of electrical pulses, no or low muscle contractions were observed with needle electrodes while strong muscle contractions were observed with surface platelets.

No serious incidents associated with pulse therapies were noted, and the majority of patients treated were willing to accept treatment again if needed.[162]

7.1.3 SIDE EFFECTS OF ELECTRIC FIELD TREATMENT IN THE HEAD AND NECK REGION

Table 7-2 displays various forms of adverse events that might occur after the treatment of mucosa in the head/throat area with pulsed electric fields. The most common event (25%) was to feel the “smell of odours”, with one serious case. Another serious side effect was “ulceration” (9%). In addition, there were less serious side effects such as “hyperpigmentation” (16%) and flu-like symptoms (14%).[227]

Table 7-2
Side effects of electric field treatment in the Head and Neck region.[227]

	Grade I/II		Grade III/IV	
Type	N	%	N	%
Odour smelling	10	23.3	1	2.3
Ulceration	4	9.3	4	9.3
Hyperpigmentation	7	16.3		
Flu - symptoms	6	14.0		
Suppuration	3	7.0		
Nausea	3	7.0		
Swelling	2	4.7		
Dysphagia	1	2.3		
Sensitive tongue, saliva stasis in the floor of the mouth	1	2.3		
Large defect of the lip, incontinency for saliva	1	2.3		

N = number of cases

7.2 THE EFFECT OF ELECTRIC PULSES ON THE FUNCTIONING OF THE HEART

7.2.1 NORMAL CONDITIONS

Figure 7-2 describes the electrophysiology of the heart with the different waveforms of the electrocardiogram (ECG) of the healthy heart. The P-wave represents the depolarization of the muscle cells in both the left and right atria from -80 mV to +20 mV, leading to a contraction in the atria. The QRS complex represents the depolarization of muscle cells in the heart's chamber also from -80 mV to +20 mV. The T-wave represents the repolarization of the muscle cells of the heart back to -80 mV.

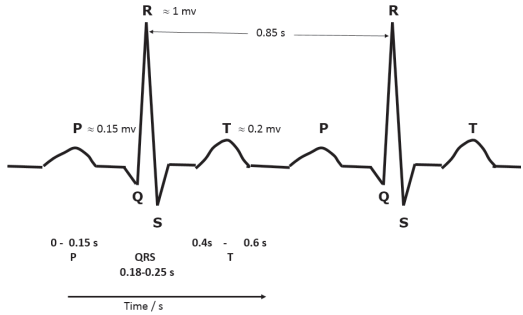

Figure 7-2
Electro-physiology of the heart. The different waveforms of the usually recorded ECG of the healthy heart.

In a study of the influence of electro-pulse-enhanced therapy on the functioning of the heart of human patients, no pathological morphological changes occurred. However, a detailed analysis of the electrocardiogram after application of electro-pulse enhanced therapy, showed a decrease in transient RR intervals, although this was not significant.[290]

Figure 7-3 displays the ratio of various ECG parameters in patients exposed to electroporation (EP) and in healthy condition (no pulses).[290]

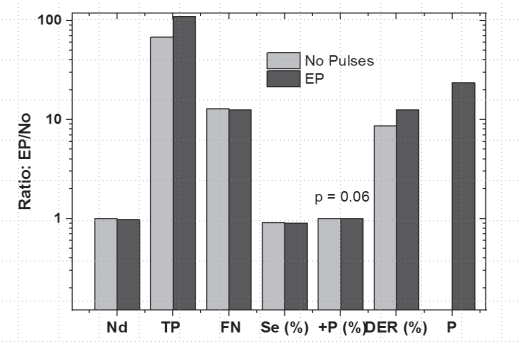

Figure 7-3
Ratio of various ECG parameters in patients exposed to electro-pulse enhanced therapy (EP) and in normal condition (no pulses)
Nd = total number of possible detected QRS complexes (normal and abnormal), the sum of TP and FN;
TP = true positive for QRS detection (the number of correctly detected QRS complexes);
FN = false negative for QRS detection (the number of missed QRS complexes);
Se = sensitivity (%) for QRS detection; %)
+P = positive predictivity (%) for QRS detection;
DER = detection error rate (%) for QRS detection;
P = premature heartbeats of ventricular, supraventricular or ectopic origin;

7.2.2 *EpEChT* THERAPY OF LIVER METASTASES

In 2015, Mali et al. studied the effects of intra-abdominal EpEChT of colorectal liver metastases on the functioning of the heart during the early post-operative care period.[291] Although the deep-seated tumours were located close to the heart, no significant heart rhythm changes (i.e., induction of extra systoles, ventricular tachycardia or fibrillation) or pathological morphological changes (i.e., ST-segment changes) indicating myocardial ischemia occurred. However, they found several minor statistically significant but clinically irrelevant changes in HRV parameters after EpEChT procedures: a decrease in median values of the mean NN interval, a decrease in the low-frequency and the normalized low-frequency component, and an increase in the normalized high-frequency component.

The conclusions here are thus that intra-abdominal *EpChT* treatment does not cause any relevant changing of the heart function. They expressed, however, statistically significant but clinically irrelevant changes in heart-rate and long-term HRV parameters. The nature of these changes is not related to the known effects of the drugs given to the patients in post-operative care.[291]

7.2.3 IRREVERSIBLE ELECTROPORATION ON THE FUNCTIONING OF THE HEART

Between September 2011 and September 2014, a single-centre nonrandomized clinical trial of irreversible electroporation (IRE) was performed at the department of surgery at Uppsala University Hospital, Sweden. The study aimed to assess the safety and efficacy in IRE ablation of 38 malignant liver tumours on 30 patients. In one patient, ECG changes occurred during IRE, and chest pain required morphine. Minor complications occurred in 6 patients, ranging from increased blood pressure to a self-limiting hematoma in the ablated zone requiring no further action.[292]

A study of demographic and intraoperative data for patients (n = 43) undergoing irreversible electroporation for *hepato-pancreato-biliary* and retroperitoneal malignancies during 2012 to 2015 was performed to describe the intraoperative adverse events associated with irreversible electroporation in patients undergoing solid tumour ablation. It revealed that there was about 20% adverse events, of which 90% were primarily cardiac, including 77% related to blood pressure elevation, and 16% to arrhythmia. One patient with arrhythmia required the termination of ablation.[293]

A study of the safety of treatment with irreversible electro-ablation involved 26 New Zealand white rabbits with VX2 tumours implanted adjacent to the sciatic nerves. After the treatment, the sciatic nerves were removed for histopathologic evaluation and showed preserved endo-neural architecture after treatment with irreversible electro-ablation.[294]

7.2.4 MICROWAVE ABLATION AND FUNCTIONING OF THE HEART

A safety and efficacy study of liver microwave (MW) ablations performed between June 2010 and August 2015 involved 118 cases of percutaneous ablation at zone margins within 5mm of the liver capsule near the heart.[295] The risk group comprised 27 cases with ablation zones extended to less than or equal to 5 mm from the myocardium, and the remaining ablation cases formed the control group.

After the treatment, there was no difference between the groups in terms of frequency of alterations in peri-procedural blood pressure (25.9% vs 29.6%. p=0.81) or heart rate (18.5% vs 24.2%. P = 0.61) or rate of LTP (12.0% vs 10.8%. P = 1.0). Thus, percutaneous MW ablation near the heart may be considered safe and effective, without increased risk of cardiac complications and with similar rates of LTP, compared with a control group of peripheral liver ablations. [295]

7.2.5 ELECTROSHOCK WEAPON ON FUNCTIONING OF THE HEART

TASER is an electroshock weapon which fires two small dart-like electrodes, which stay connected to the central unit by conductors, to deliver an electric shock to voluntary disrupt control of muscles, resulting in strong involuntary long muscle contractions.

Table 7-3
Specifications of electroshock weapon TASER type M26 and X26

Specification	Taser M26	Taser X26
Output pulse amplitude	3800 V	600 V
Pulse length	40μs	100μs
Pulses delivered in a shot	100	95
Duration	5 s	5 s
Pulse delivery frequency	20 Hz	19 Hz

Table 7-3 shows the specification of the TASER X26 pulse, which is very similar to the pulses from an electroporation device. The probability of triggering cardiac arrhythmias in the use of electroshock weapon depends on the currents that occur in the heart and surrounding organs.[296,297]

A single coarse mesh finite element model using the time-duration threshold of approximately $2.98 \cdot 10^{-3}$ (V×/m) for nerve stimulation, shows that TASER would not stimulate regions farther than roughly 19 cm away from the darts.[297]

Thus electroporation devices that deliver pulses with a short duration and low duty cycle similar to TASER should not cause any risk of triggering cardiac arrhythmias (VF) at treatment locations about 20 cm away from the heart.

7.3 SAFETY AND EFFICACY OF IRREVERSIBLE ELECTROPORATION (IRE)

7.3.1 IRE ABLATION OF LIVER TUMOURS

In irreversible electroporation (IRE), the applied field-strength is higher than in electro-chemotherapy in order to kill (ablate) the tumour cell instantly without adding any chemotherapy drugs. Thus, the safety matter of IRE has been carefully studied in several clinical trials.

A single-centre nonrandomized clinical trial of the safety and efficacy of IRE ablation involved 30 patients with 38 liver tumours.[292] At six months after the IRE treatment, there was no evidence of a tumour in the ablated area in 66% of the patients. In six patients, minor complications appeared, and, in one patient, bile duct dilatation and stricture of the portal vein and bile duct occurred.

All publications reporting clinical responses from IRE ablation of liver tumours demonstrate the need for the conducting of more clinical studies in order to establish full safety and efficacy for IRE treatment of primary and metastatic hepatic malignancies.[298]

7.3.2 NERVES ADJACENT TO IRE ABLATION OF TUMOURS

A study of the safety of IRE ablation on the sciatic nerve involved 26 New Zealand white rabbits VX2 with tumours implanted adjacent to the sciatic nerves.[298] After IRE ablation, severe damage appeared on nerves adjacent to the tumour. However, in a short time, their function and structure repaired. The control rabbits also showed nerve damage but with no signs of repair and rapid tumour progression. Thus, irreversible electro pulse

treatment of tumours located adjacent to nerves seems not to cause severe nerve damage.

CHAPTER VIII

NEW DIMENSIONS FOR ELECTRO-PULSE-ENHANCED CANCER-THERAPY

8.1 CLINICAL *EpECT* STUDIES

8.1.1 RECENT CLINICAL *EpECT* STUDIES

Most clinical studies so far have used the ESOPE protocol with pulses of constant voltage amplitude applied in a train of 8 pulses at an interval of 0.2ms. By successively decreasing voltage for each pulse, a new treatment concept called dynamic electro-enhanced chemotherapy (D-EECT™) developed. A clinical case study in India recently applied the D-EECT concept.[178] The study involved 23 patients treated in a total of 38 sessions of D-EECT™ at four different cancer centres. Table 8-1 shows the treatment responses for a total of 66 tumours of various sizes and various histological types. In all of the evaluable tumours, the objective responses were found to be 100% with complete remissions 18%, and partial remissions 82%. No progressive disease was recorded and the average weighted (WR_4) response became 58 ± 4(SE)%.

The objective responses of 100% squamous cell carcinomas in Kalavathy's study are better than the overall average of 78% for the objective response estimated from all reported clinical studies of SCC reviewed in Chapter 6. The weighted (WR_4) response of 61% was greater than the overall average of 48% for the weighted (WR_4) response estimated from all reported clinical studies of SCC reviewed in Chapter 6. No cases with progressive disease appeared in Kalavathy's study, which might indicate a better immune response than the clinical studies of SCC reviewed in Chapter 6 with an estimated fraction progress disease of 12%.

Table 8-1
Type of tumours and the percentage of tumours' response to D-EECT treatment.[178]

Tumour Type	Histology	CR %	PR %	OR %	WR_4 %
Squamous cell ca.	HN SCC	21	79	100	61
Breast cancer	Adeno carcinoma	14	86	100	57
Breast cancer	Infiltrated ductal	50	50	100	75
Breast cancer	Poorly diff.	0	100	100	50
Rectal cancer	Adeno carcinoma	0	100	100	50
Vaginal vault ca.	Squamous	0	100	100	50
Soft tissue sarcoma		0	100	100	50
Fibro sarcoma		67	33	100	84
Spindle cell ca.		0	100	100	50
Average		18	82	100	58
SE		8	8		4

Table 8-2 shows the summary of another recent report of 43 patients with recurrent mucosal head and neck tumours treated according to the ESOPE protocol.[227] A cohort of 34 patients had evaluable SCC tumours, and 3 were adenocarcinoma patients. The SCC patients were split into one group with nodule diameter less than 3 cm, and another group with nodules larger than 3 cm. Table 8-2 shows the summary of the averages of the various responses of the total number of patients and the two groups separately.

There was no remarkable variation of responses in the various groups of patients shown in Table 8-2, except that the weighted (WR_4) response was greater in the group with small tumours than in the group with large tumours. In Kalavathy's work, however, the size of the SCC nodules was in the range of 5-6 cm with 61% weighted (WR_4) response, which is greater than corresponding value of 38% for tumours larger than 3 cm treated according to the ESOPE protocol.[178]

Table 8-2a
Summary of the results of EpEChT according to ESOPE in treatment of head and neck cancer.[227]

Type of patients	No. of patients	No. patients evaluable	fCR	fPR	fSD	fPD
All	43	37	0.22	0.43	0.27	0.08
All SCC 91%		34	0.21	0.44	0.26	0.09
SCC<3 cm		14	0.21	0.50	0.21	0.07
SCC>3 cm		20	0.20	0.40	0.30	0.10
Tot AD 9 %	4	3	0.2	0.4	0.3	0.1

Table 8-2b
Summary of objective OR and weighted WR_4 treatment response to EpEChT according to ESOPE in treatment of head and neck cancer.[227]

Type of patients	No. of patients	No. patients evaluable	fOR	fWR4
All	43	37	0.65	0.42
All SCC 91%		34	0.65	0.41
SCC<3 cm		14	0.71	0.44
SCC>3 cm		20	0.60	0.38
Tot AD 9 %	4	3	0.6	0.38

The objective responses of 100% for breast cancer in Kalavathy's study are better than the overall average of 80% for the objective response estimated from all reported clinical studies of SCC reviewed in Chapter 6. However, the ratio of complete to partial remissions is 0.25 in Kalavathy's study, which is much smaller than the ratio of 2 for corresponding averages estimated from all reported clinical studies of breast cancer reviewed in Chapter 6.

Table 8-3 shows a summary of another recent report on electro-pulse enhanced chemotherapy treatment of 90 patients with cutaneous recurrence from breast tumours according to the ESOPE protocol at 10 European cancer centres. The table shows the response to electro-pulse-enhanced chemotherapy for breast tumours according to the size of nodules less than 3cm in size, or larger than 3cm, and at the bottom of the table the average patient response at 2 months after treatment is given.[236] The weighted (WR_4) response was 80% in the group with small tumours and 48% in the group of large tumours.

Table 8-3a
Tumour response to electro-chemotherapy according to ESOPE for a size of nodules < 3 cm, and > 3cm, and the average patient response at 2 months after treatment.[236]

	No. Nodules	fCR	fPR	fSD	fPD	fNE
Nodules < 3 cm	113	0.72	0.14	0.08	0.01	0.05
Nodules > 3 cm	94	0.37	0.24	0.18	0.06	0.03
Average	207	0.57	0.20	0.13	0.03	0.04

fNE is the fraction of patients not included in the evaluation.

Table 8-3b
Summary of objective OR and weighted WR_4 tumour response to electro-chemotherapy according to ESOPE for a size of nodules < 3 cm, and > 3cm, and the average patient response at 2 months after treatment.[236]

	No. Nodules	fOR	fWR_4
Nodules < 3 cm	113	0.86	0.80
Nodules > 3 cm	94	0.62	0.48
Average	207	0.76	0.67

In Kalavathy's study[178]of breast cancer patients with nodules in the range of 7-8 cm, the weighted response is about the same as it is for tumours larger than 3 cm in the ESOPE study. Her treatment of patients with breast-infiltrated ductal carcinoma with even larger tumours (10-15 cm) resulted in a weighted (WR_4) response of 75%, which is better than the 48% weighted (WR_4) response for tumours larger than 3cm in the ESOPE study.[236]

8.1.2 *EpEChT* AND CYCLOPHOSPHAMIDE (CTX)

Cyclophosphamide has been found to induce beneficial immunomodulatory effects in adaptive immunotherapy with the following mechanisms involved in the creation of this immunologic effect.[116]

1. Elimination of $CD4^+$, and $CD25^+$ T-cells in naive and tumour-bearing hosts;
2. Induction of T- cell growth factors such as type-I interferons (IFNs);
3. Enhanced grafting of adoptively transferred, tumour-killing effector T-cells (CTL).

Thus, cyclophosphamide preconditioning of recipient hosts (for donor T-cells) might be used to enhance immunity in naïve hosts and to enhance adaptive immunotherapy.

A recent in vitro study showed that electro-enhanced chemotherapy with cyclophosphamide (CTX) has a similar anti-tumoural effect as Bleomycin.[117]

Kalavathy successfully treated one patient with locally advanced breast cancer, but with no secondary deposits anywhere, by using the routine chemotherapy combination of Adriamycin, and Cyclophosphamide (with a dose according to the BSA) followed by *EpEChT* within 24 hours of intravenous administration of chemotherapy drugs.[178]

The combination of electro-enhanced gene therapy with Vasostatin-gene and cyclophosphamide administration was found to improve the therapeutic effects in melanoma tumours. Both significant inhibition of tumour growth and extended survival of treated mice were observed.[118]

8.1.3 *EpEChT* AND TAMOXIFEN?

Studies of MCF-7 human breast adenocarcinoma cells with tamoxifen in combination with pulsed electric fields resulted in enhanced uptake of Tamoxifen and decreased tumour cell growth compared to treatment with tamoxifen alone.[299,300] These results suggest that clinical use of electro-pulse-enhanced therapy combined with tamoxifen could lead to a more effective anti-tumour response than tamoxifen alone.[299] Reducing Tamoxifen dosage for the treatment of Estrogen receptor-positive breast cancer might also become a consequence of treatment with pulsed electric fields.[300]

8.2. ELECTRO-PULSE ENHANCED IMMUNO THERAPY

8.2.1 *EpEChT* AND INTERLEUKIN-2 (IL-2)

In animal experiments conducted by Mir and colleagues in 1992, systemic, anti-metastatic immune responses were achieved through a combination of electro-pulse enhanced chemotherapy and delivering histo-incompatible cells secreting interleukin-2 (IL-2).[164,120,301,302,119]

Later, Heller discussed the clinical potential of the use of electro-pulse enhanced gene-therapy (*EpEGT*) in the treatment of melanoma.[303] Delivery of a plasmid DNA encoding interleukin-12 (IL-12) or interleukin-2 (IL-2) using EpEGT proved to be safe with no severe toxicities reported, while EpEGT with the delivery of IL-12 plasmid DNA resulted in significant necrosis of melanoma cells in the majority of treated tumours. Biopsies from

the treated tumours showed significant lymphocytic infiltration. Following this treatment, a systemic response in untreated melanoma lesions also appeared.[303]

In a recent review, Calvet and Mir (2016) noted the promising outcome of combining electro-enhanced chemotherapy with immunotherapy.[124] By the end of 2015, about 140 cancer treatment centres in Europe practiced electro-pulse-enhanced chemotherapy for local treatment of skin metastases in about 13,000 cancer patients. By combining DNA vaccination and cytokine-based immunotherapy to stimulate anti-tumour immunity in combination with electro-pulse enhanced chemotherapy, a systemic anti-metastatic response of clinical significance might occur.[124]

8.2.2 *EpECT* AND IMMUNOMODULATING ANTIBODIES (CTLA-4 AND PD-1)

The use of the anti-CTLA-4 drug Ipilimumab and the anti-PD-1 drug Nivolumab in patients with advanced melanoma has been shown to improve progression-free survival and objective response. However, the use of these drugs might cause such severe toxicity that some patients discontinue the treatment.[281]

A retrospective multicentre program to study a combination of electro-pulse-enhanced chemotherapy according to the ESOPE protocol with 15000 IU Bleomycin, and immune therapy using either the anti-CTLA-4 drug Ipilimumab or the anti-PD-1 drugs Nivolumab or Pembrolizumab, involved 33 patients with distant cutaneous melanoma metastases.[304]

Table 8-4 shows the summary of responses of the various treatments derived from the published data. The local objective (OR) and weighted (WR_4) responses of the combination are, however, much lower than those for EpEChT alone, as reviewed in Chapter 6. The systemic responses of the combination are quite low, and due to common progressive disease (PD), the weighted (WR_4) response is strongly negative for Ipilimumab and close to zero for anti-PD-1. The toxicity of Ipilimumab in the combined treatment was found to be more severe than it was for treatment using Ipilimumab alone.[304]

A case of massive skin metastasis from melanoma that relapsed after repeated electro-pulse enhanced chemotherapy, received further treatments with checkpoint immune therapy. At 6 months after treatment with anti-PD-1 Nivolumab, the complete response appeared.[305]

Another case with a relapsed progressive mass of malignant melanoma was treated with electro-pulse-enhanced chemotherapy using an IGEA Cliniporator device through a hexagonal electrode on the progressive mass,

while on anti-PD-1 Nivolumab treatment. Complete response was achieved, with no evidence of disease at 4 years after the local recurrence.[284]

Table 8-4a
The response of combining electro-pulse-enhanced chemotherapy and checkpoint immune therapy.[304]

Drug	Response	No. patients	CR	PR	SD	PD
Ipilimumab	Local	28	0.18	0.50	0.11	0.21
Ipilimumab	Systemic	26	0.08	0.12	0.08	0.73
anti-PD-1	Local	5	0	0.09	0	0.06
anti-PD-1	Systemic	5	0	0.06	0	0.09

Table 8-4b
Summary of objective OR and weighted WR_4 response of combining electro-pulse-enhanced chemotherapy and checkpoint immune therapy.[304]

Drug	Response	No. patients	OR	WR_4
Ipilimumab	Local	28	0.68	0,25
Ipilimumab	Systemic	26	0.19	-0,57
anti-PD-1	Local	5	0.09	-0,02
anti-PD-1	Systemic	5	0.06	-0,06

The contradictory results of the multicentre study and the case reports require more studies to examine the timing and dosage of immunomodulation antibodies in combination with electro-pulse therapy.

8.4 ELECTRO-PULSE ENHANCED RADIATION THERAPY "*EPERT*"

8.4.1 *EPERT* OF FIBROBLAST CELLS AND SUBCUTANEOUS IMPLANTED TUMOURS IN RAT

Radiation therapy alone is known to have limited therapeutic effects on brain tumours, in contrast to when it is used in combination with high voltage pulses. Effective killing of V-79 fibroblast cells remarkably occurred after combined exposure with 2Gy gamma-radiation and exponential shaped pulsed electric fields with an amplitude of 1600V/cm and a time-constant of 1ms at 1Hz repetition.[128,77,129,130]

The encouraging results from the experiments with V79-cells raised the idea of combining treatment of pulsed electric fields and gamma-radiation in Fischer rats with N32 tumours implanted on their flanks.

Each experiment was performed on four different groups with 6 animals in each:

- Untreated Controls (Ctrl);
- Radiation therapy only (RAD);
- Pulsed electric field only (PEF);
- Combined radiation therapy and pulsed electric field, (RAD + PEF)

Lead blocks surrounded the tumour to focus the radiation from the source at a distance (SSD) of 80 cm. A pulsed electric field applied by an isolated slide-calliper with plane electrodes (15x15 mm) was mounted on the blades enclosing the tumour. The tumour volume was estimated as an ellipsoid by measuring the length, width, and thickness with the slide-calliper.

A dramatic decrease of the tumour volume appeared on subcutaneously implanted tumours through the combed treatment of pulsed electric fields and gamma-radiation. Since the radiation dose used in the combined treatment is low (15-30Gy), it might be possible to treat recurrent tumours which already received a full dose with conventional radiation therapy. It is, thus, worthwhile to further explore this combined type of treatment. It might be of particular interest to apply high voltage impulses combined with brachy-therapy where the radioactive source (e.g. Iridium-192) can serve as an electrode.[133]

8.4.2 EpEChT COMBINED WITH RADIATION THERAPY

EpECT of EAT tumours in mice with Cisplatin and RT

Sersa and colleagues performed a study of combined modality therapy with Cisplatin and radiation using electroporation of subcutaneous Ehrlich ascites tumours (EATs) in CBA mice.[134,135] The mice were treated either by Cisplatin (CDDP), electric pulses (EP), or ionizing radiation (RT). Figure 8-1 displays the results of the single (CDDP 4 mg/kg body weight, EP 8 pulses of 1300V/cm and 0.1ms, RT 15 Gy single fraction), and combined treatments (EP+CDDP, EP+RT, RT+CDDP, and EP+CDDP+RT) recorded 100 days after treatment. These results show that delivery of Cisplatin into the cells through electroporation of tumours increases the radio-sensitizing effect of Cisplatin. However, the enhanced therapeutic effect may also be due to radiation-reduced tumour immunosuppression.

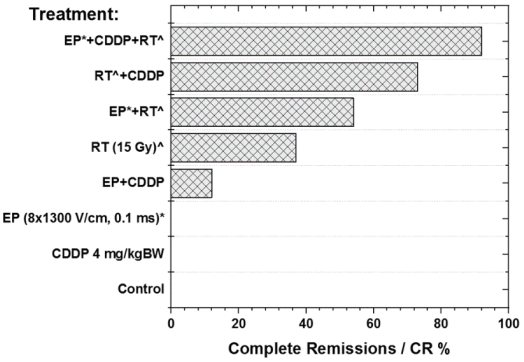

Figure 8-1
Treatment results at 100 days after the various types of treatment.[134,135]

EpEChT of LPB sarcoma in mice with Cisplatin and RT

In a 2003 study, Kranjc and collaborators treated LPB murine sarcoma cells and tumours in mice, either with Cisplatin, electroporation or ionizing radiation alone, as well as combinations of these. In vitro response was determined by colony forming assay, while in vivo treatment effectiveness was determined by the values of the 50% tumour control dose (TCD50).

They found that exposure of cells in vitro to a combination of Cisplatin and electroporation increased accumulation of Cisplatin, and reduced tumour perfusion. In combination with ionizing radiation, the treatment effect was significantly enhanced by 60%, compared to irradiation only and by 40% compared to tumours treated with Cisplatin and irradiation.[136]

EpEChT of LPB sarcoma in mice with Bleomycin and RT

In a 1995 study, Okunieff and colleagues studied the effect of EpECT using Bleomycin combined with radiation therapy on subcutaneous murine sarcoma LPB cells, syngeneic to the C57Bl/6 mice used in the experiments. Three minutes after intravenous injection of Bleomycin (BLM) in a volume of 150 µl, they exposed a tumour to 8 electric pulses of 1300 V/cm with 0.1ms duration at 1 Hz, using plate electrodes placed on the skin overlying the tumour.[137]

Table 8-5 shows the values of the TCD50 for various combinations of treatment, and the relative tumour control (RTC), as opposed to radiotherapy only (RT). Neither treatment of animals with radiation alone (RT) nor the application of electric pulses or Bleomycin on the tumours before irradiation (EpE+RT and Bleo+RT) had any effect on the TCD50. However, the application of electric pulses with Bleomycin simultaneously combined with radiation therapy (EpEBleo+RT), decreased the TCD50

value of tumours by a factor of 1.9 compared to any other treatment. Hence, it is evident that radiation therapy of tumours significantly contributed to the therapeutic effect of EpEChT on tumours with BLM.[138]

Table 8-5

The values of the 50% tumour control dose (TCD_{50}) and the relative tumour control for various combinations of treatment.[138]

Treatment	TCD_{50}	SD	RTC
RT	23.1	0.3	1.0
EpE+RT	22.1	0.3	1.0
Bleo+RT	22.8	0.3	1.0
EpEBleo+RT	12.4	0.3	1.9

EpECT using Bleomycin and RT

As shown in the previous paragraph, radiation therapy two-fold increased the therapeutic effect of electro-chemotherapy with Bleomycin.[138] Further studies in two murine tumour models with different histology showed that electro-pulse-enhanced chemotherapy before radiotherapy of the tumours and a single fraction of ionizing radiation of 10 Gy reduced the tumour volume by 98%, and five daily fractions of 2 Gy reduced the volume by 85%.[139]

The mechanism behind the effect of the combination of EpECT and RT is probably similar to the enhancement of the effect of tumour vaccine achieved by a single fraction of radiation therapy (RT).[143,142,141,306] The creation of a bolus of dead tumour cells as a result of the EpECT treatment is similar to the administration of a bolus of dead tumour cells that activates the tumour immune-suppression by an accumulation of Myeloid-derived suppressor cells (MDSC) in the tumour microenvironment. A single fraction of ionizing radiation of about 8 Gy deactivates the immune-suppressive effect over 10-14 days. If there are surviving tumour cells present after the treatment, the tumour starts growing again. Thus, a few repeated treatment regimens are required to achieve a complete cure.

The results of the combination of EpEChT and radiation therapy suggest a therapy regime with repeated treatments of EpEBleo. + RT (6-10 Gy) once a week until complete remission occurs. The absorbed dose and number of treatments required might vary with the histology of a tumour and from patient to patient. To bring this therapy regime to clinical practice is a challenge to the oncology community with clinical trials and efforts to standardize the treatment parameters.

8.5 IRREVERSIBLE ELECTROPORATION IRE

Irreversible electroporation (IRE) is a technique for the treatment of tumours with high-voltage (2-3 kV/cm) electrical pulses.[307] In liver and pancreatic cancer surgery, irreversible electroporation IRE is used as an alternative to the more established method of HF thermal ablation.

Table 8-6
Results from five clinical studies of liver cancer (CRLM: colorectal liver metastasis. HCC: hepatocellular carcinoma) treatment with IRE.

Reference	HCC	CRLM	Other	CR	Com	FT
				%	%	
	No.	No.	No.			
Cannon,2013[309]	14	20	10	97	9	12
Cheung,2013[310]	18			67	34	18
Kingham,2012[311]	11	21	5	96	14	6
Scheffer,2014 [312]		10		90	0	1
Sugimoto,2015[313]	6			83	0	9
Average ± SD				87±12	11±11	9

CRLM: colorectal liver metastasis; HCC: hepatocellular carcinoma
CR: Primary efficiency, Com: Complications, FT: Followup time in months

The IRE method has similar treatment results to thermal ablation, but has the advantage of avoiding severe thermal complications.[308]

Table 8-6 shows that the mean total remissions in five clinical studies of liver cancer treatment with IRE are 87±12%.[309,310,311,312,313] The levels of local recurrence and minimal complications are both, on average, about 11% at a follow-up time of around 9 months.

IRE treatment of pancreatic cancer results in increased survival time but with more complications compared to the treatment of liver cancer. Table 8-7 shows the results of 5 clinical studies,[314,315,316,317,318,319] and one single patient.[314] Despite the risk of significant complications, the IRE is considered to be a possible alternative for the treatment of pancreatic cancer.[319]

However, clinical randomized studies and development of treatment protocols are required to establish the method in clinical routine.[308]

Table 8-7
Results from five clinical studies of pancreatic treatment with IRE.

Author[Reference]	Patient No.	CR %	Com. No.	FT
Bagla,2012[314]	1	100	0	6
Martin,2012[315]	27	96	4	3
Martin,2013[316]	54	94	32	12
Narayanan,2012[317]	14	100	4	14
Paiella,2015[318]	10	100	3	15
Sugimoto, 2018[319]	8	NR	6/8	12[*)]

CR: Primary efficiency, Com: Complications FT: Follow-up time in months
NR: not reported, [*)]Progression free survival time

Sano reported in 2018 of an *in vitro* experiment on cell cultures, combining IRE treatment with 500 ns pulses and radiation therapy in single dose fractions of 2 or 20Gy.[320] IRE and radiation therapy (RT) appear to induce different delayed cell death mechanisms. After 14 days, the combinatorial therapy resulted in the lowest cell survival. These results are in agreement with the findings reported above (8.4), and indicate an *in vivo* antitumor efficacy of combined H-FIRE and RT as well.[320]

8.6 NANOSECOND HIGH VOLTAGE ELECTRIC PULSES

High voltage electric pulses of nanosecond (1ns $=10^{-9}$ s) pulse length interact with several biological targets, such as plasma membranes, intracellular structures, calcium ions, cell nuclei, and mitochondria. A review of the effects of ns-pulses on eukaryotic cells summarizes and analyses the published data for assessing the use of ns-pulses in electroporation therapy.[321]

A recent study of the effect of ns-pulses on the inhibition of oral cancer growth in vitro and in vivo revealed their potential use in oral cancer therapy.[322] However, the most intriguing effect of ns-pulse treatment is the eradicating of local breast cancer while reducing distant metastases recently demonstrated by Guo (2018).[323] A single ns-pulse treatment with 600 pulses, with a field strength of 50 kV/cm and 100ns pulse length at 1Hz, results in complete regression of a poorly immunogenic, metastatic 4T1-Luc mouse mammary carcinoma. Secondary tumour cell challenge demonstrated a vaccine-like effect of the ns-pulse treatment, which suggests a potent antitumor immunity effect on distant metastases in addition to a local tumour eradication.[323]

8.7 CONCLUSION *EpECT*

In summary, there are great clinical potentials in applying electro-pulse treatment to enhance the effect of established cancer therapy regimes.

8.7.1 CHEMOTHERAPY *EpEChT*

Electro-pulse-enhanced chemotherapy (EpEChT), as reviewed in Chapter 6, is already clinically established in the treatment of tumours. However, with further experience, and with improvements such as the development of new drugs, pulse-sequences and protocols, we should be able to approve the EpEChT method treatment for primary tumours.

8.7.2 RADIATION THERAPY *EpERT*

Preclinical studies indicate excessive potential in a combination of electro-pulse treatment and radiation therapy.[142,143]

A few clinical studies have been performed with the combination of electro-pulse-enhanced chemotherapy and radiation therapy, with great success.[138,139]

8.7.3 IMMUNE THERAPY *EpEIT*

DNA vaccination and cytokine-based immunotherapy stimulate anti-tumour immunity which, in combination with electro-pulse-enhanced chemotherapy, creates a systemic anti-metastatic response with vast clinical potential. The use of EpECT in combination with immune-modulating antibodies (CTLA-4 and PD-1), however, results in clinical outcomes which require more studies to examine the timing and dosage. In summary, there is great clinical potential in applying electro-pulse treatment to enhance the effect of established cancer therapy regimes. However, the best regime appears to be the combination of electro-pulse enhanced immunotherapy combined with a single fraction of radiation therapy. [142,141,306,143]

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