University of Ljubljana
Faculty of Electrical Engineering

Barbara Mali

The safety and effectiveness of electrochemotherapy

doctoral thesis

Mentor:
Assoc. Prof. Tomaž Jarm, Ph.D.

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Fakulteta za elektrotehniko

Barbara Mali

Varnost in učinkovitost elektrokemoterapije

doktorska disertacija

Mentor:
izr. prof. dr. Tomaž Jarm

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PREFACE

The thesis addresses the following interrelated topics: the effects of electrochemotherapy on functioning of the heart, the development of algorithm for synchronization of electroporation pulses with electrocardiogram, and of the role of different treatment conditions on effectiveness of electrochemotherapy.

The results presented in the thesis are based on the following papers:

PAPER I: **Mali B**, Jarm T, Corovic S, Paulin-Kosir MS, Cemazar M, Sersa G, Miklavcic D. The effect of electroporation pulses on functioning of the heart. *Med Biol Eng Comput* 46(8): 745-757, 2008.

PAPER II: **Mali B**, Sersa G, Miklavcic D, Jarm T. Early effects of intra-abdominal electrochemotherapy of tumors in liver on functioning of the heart. *In preparation for submission.*

PAPER III: **Mali B**, Sersa G, Miklavcic D, Jarm T. Late effects of intra-abdominal electrochemotherapy of tumors in liver on functioning of the heart. *In preparation for submission.*

PAPER IV: Edhemovic I, Gadzijev E, Brecelj E, Miklavcic D, Kos B, Zupanic A, **Mali B**, Jarm T, Pavliha D, Marcan M, Gasljevic G, Gorjup V, Marolt-Music M, Pecnik-Vavpotic T, Cemazar M, Snoj M, Sersa G. Electrochemotherapy: a new technological approach in treatment of metastases in the liver. *Technol Cancer Res Treat* 10(5): 475-485, 2011.

PAPER V: **Mali B**, Jarm T, Jager F, Miklavcic D. An algorithm for synchronization of *in vivo* electroporation with ECG. *J Med Eng Technol* 29(6): 288-296, 2005.

PAPER VI: **Mali B**, Jarm T, Snoj M, Sersa G, Miklavcic D. Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis. *Accepted for publication in EJSO.*

PAPER VII: **Mali B**, Miklavcic D, Campana LG, Cemazar M, Sersa G, Snoj M, Jarm T. Tumor size and effectiveness of electrochemotherapy. *Submitted to Radiol Oncol.*

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ABSTRACT

Electrochemotherapy (ECT), a local treatment of tumors, is gaining important role in clinics. The use of ECT on cutaneous and subcutaneous tumors in clinics is increasing due to favorable treatment characteristics that are: high effectiveness, safety, simplicity of application, normal-tissue- and organ-sparing property, low toxicity, possible application in an out-patient setup, cost-effectiveness, and suitability for repetitive and neoadjuvant treatment. However, new ECT modalities with surgical, endoscopic or percutaneous approaches are being developed for treatment of deep seated tumors, which however may potentially increase the risk of undesirable side effects to the patient. Although ECT is considered safe and highly effective, some details regarding the safety and effectiveness of ECT are still not fully understood and there is still room for further optimization of the procedure.

The aims of this doctoral thesis cover three important issues concerning clinical use of ECT: (i) evaluation of safety of clinical ECT, in view of its potential influence on functioning of the heart; (ii) development and evaluation of algorithm for synchronization of electroporation pulse delivery with electrocardiogram (ECG); and (iii) evaluation of effectiveness of clinical ECT and its dependence on treatment conditions.

The safety aspect of clinical ECT was evaluated using ECG signals recorded during ECT of cutaneous and subcutaneous tumors, and before, during and after intra-abdominal ECT of tumors in liver. The results show that there are several early (occurring during and immediately after ECT treatment) and late (occurring within 24 hours after ECT treatment) effects of ECT on functioning of the heart, but no adverse effects were detected. Statistically significant early effect, manifested as transient decrease in RR and QRS interval duration (i.e. increase in heart rate) was induced during unsynchronized electroporation pulse delivery on cutaneous and subcutaneous tumors, which can be largely (if not completely) attributed to anxiety and stress of the patient undergoing ECT procedure. In addition, the results of analysis of ECG signals recorded during intra-abdominal ECT of tumors in liver demonstrate an early effect of administration of bleomycin, expressed as occurrence of premature atrial contractions immediately during intravenous administration, and an early effect of synchronized electroporation pulse delivery, expressed as transient decrease in duration of

the corrected QT interval and increased short-term heart rate variability (HRV). This early effect appeared most likely due to effect of electric stimulation of surrounding muscles and nerves caused by electroporation pulses, which consequently provoke cardiovascular responses, commonly manifested as transient increase in heart rate. Furthermore, the results of HRV analysis of ECG signals recorded before and after intra-abdominal ECT of tumors in liver show late effects, expressed as increased heart rate (i.e. decreased NN interval) and decreased long-term HRV parameters SD2 and LF. Currently, it is not clear however whether these detected changes in HRV appear due to ECT procedure alone or due to effects of post-operative pain and drugs administered in post-operative care.

Our published study on effects of ECT of cutaneous and subcutaneous tumors on functioning of the heart was the first study, in which the need for synchronization of electroporation pulses with ECG was addressed, in order to maximize the safety of the patients. This issue was at that time particularly important for the future clinical studies using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or for studies including treatment of deep seated tumors in the immediate vicinity of the heart. Nowadays, synchronization of electroporation pulse delivery with ECG is routinely used in treatment of deep seated tumors.

We developed an algorithm for synchronization of electroporation pulses with ECG. The evaluation of the algorithm on ECG signals from standard database and on ECG signals recorded during clinical ECT demonstrates that our algorithm enables safer use of ECT. The algorithm allows delivery of electroporation pulses only outside the vulnerable period of the ventricles and prevents pulses from being delivered in case of the appearance of heart arrhythmias, such as atrial and ventricular premature beats. We demonstrated that the algorithm presents an important improvement over the currently used protocol for synchronization implemented in clinical device for ECT, particularly for treatment of deep seated tumors located close to the heart. The algorithm could be used for effective and reliable synchronization of electroporation pulses with ECG for use in all medical applications that include delivery of high-voltage pulses (like in ECT, gene electrotransfection and irreversible electroporation techniques) regardless of tumor location.

In order to consolidate current knowledge and experience on ECT treatment from the effectiveness point of view, we performed a systematic review of the literature regarding

clinical ECT. Up-to-date overall effectiveness of single-session ECT of cutaneous and subcutaneous tumors, regardless of different treatment conditions and parameters used, was evaluated as complete response rate of 59.4% and objective response rate of 84.1%. The results of the analysis show that electroporation pulse delivery significantly potentiates the effectiveness of chemotherapeutic drug alone by more than 50%. The differences in effectiveness of single-session ECT were found statistically significantly dependent on several treatment conditions, i.e. on chemotherapeutic drug, tumor histotype and tumor size. The results of our systematic review shed new light on effectiveness of ECT and can be used for prediction of tumor response to ECT with respect to various treatment conditions and should be taken into account in further refinement of ECT protocols on cutaneous and subcutaneous tumors as well as in development of ECT procedures for treating deep seated tumors.

RAZŠIRJENI POVZETEK

OZADJE ZNANSTVENEGA PODROČJA

ELEKTROPORACIJA

Če celico, skupek celic ali tkivo izpostavimo električnemu polju, se na celični membrani inducira transmembranska napetost, ki je sorazmerna električnemu polju. Ta napetost se prišteje k mirovnemu potencialu membrane, kar povzroči spremembe v skupni transmembranski napetosti membrane [Kotnik et al. 1997, Teissie et al. 1999, Miklavcic et al. 2000]. Ko skupna transmembranska napetost preseže pragovno vrednost (nekje med 200 mV in 1 V), se v celični membrani pojavijo strukturne spremembe v obliki hidrofilnih por, ki znatno povečajo prepustnost membrane [Neumann et al. 1982, Weaver & Chizmadzhev 1996, Weaver 2000, Kotnik et al. 2010]. Ta pojav imenujemo elektropermeabilizacija oziroma elektroporacija. Povečana prepustnost celične membrane omogoči molekulam, ki jih membrana v fizioloških pogojih ne prepušča, da preidejo v notranjost celice [Mir et al. 1991b]. Glede na izbrane električne pogoje (število, oblika, amplituda, trajanje in ponavljalna frekvenca električnih pulzov ter smer električnega polja) in lastnosti celice oziroma tkiva lahko dosežemo bodisi reverzibilno bodisi ireverzibilno elektroporacijo [Macek Lebar et al. 2002, Zupanic et al. 2008, Cemazar et al. 2009, Miklavcic & Towhidi 2010, Miklavcic et al. 2010, Mir 2000, Davalos et al. 2005, Al-Sakere et al. 2007]. Pri reverzibilni elektroporaciji uporabljajo take električne pogoje, da se elektroporirana celična membrana lahko povrne v prvotno stanje in tako celica preživi, ireverzibilno elektroporacijo pa izvajajo pod takimi pogoji, ki povzročijo trajno elektroporacijo celične membrane in s tem celično smrt.

ELEKTROKEMOTERAPIJA

Reverzibilna elektroporacija se trenutno največ uporablja v kliniki za zdravljenje tumorjev s t.i. elektrokemoterapijo (ECT – angl. electrochemotherapy). ECT je kombinirano zdravljenje tumorjev, pri katerem intravenskemu ali intratumorskemu injiciranju kemoterapevtika sledi dovajanje visokonapetostnih električnih pulzov (elektroporacijskih pulzov) lokalno na tumor. Elektroporacijski pulzi povzročijo elektropermeabilizacijo celične membrane, kar omogoči vstop hidrofilnim protitumorskim učinkovinam, kot sta bleomicin in cisplatin, v tumorske celice in s tem poveča učinkovitost zdravljenja tumorjev [Mir et al. 1991b, Sersa et al. 1995]. Poleg neposrednega delovanja citostatikov na tumorske celice ima

ECT tudi posreden žilno-razdiralni učinek zaradi delovanja citostatikov na endotelne celice tumorskega žilja, učinek na zmanjšanje pretoka v tumorskem tkivu, kar pripomore k večji učinkovitosti zdravljenja zaradi podaljšanega zadrževanja kemoterapevtika v tumorskem tkivu, in učinek na aktivacijo imunskega sistema pacienta [Sersa et al. 2008a, Jarm et al. 2010, Sersa et al. 1997, Daud et al. 2008, Cemazar et al. 2010].

Zaradi enostavnega fizikalno-kemičnega principa ECT, ki teoretično lahko permeabilizira celice kateregakoli tipa, pričakujemo, da z ECT lahko dosežemo dobre učinke zdravljenja tumorjev kateregakoli histološkega izvora. Učinkovitost klinične ECT so do sedaj pokazali pri zdravljenju kožnih in podkožnih tumorjev različnih histoloških izvorov, na primer malignega melanoma, ploščato-celičnega karcinoma glave in vratu, bazalno-celičnega karcinoma, Kaposijevega sarkoma in adenokarcinoma dojke [Byrne et al. 2005, Gaudy et al. 2006, Larkin et al. 2007, Kis et al. 2011, Quaglino et al. 2008, Sersa et al. 2000, Snoj et al. 2007, Allegretti & Panje 2001, Bloom & Goldfarb 2005, Burian et al. 2003, Gargiulo et al. 2010, Landstrom et al. 2010, Sersa et al. 1998, Curatolo et al. 2008, Curatolo et al. 2012, Garbay et al. 2006, Whelan et al. 2006, Testori et al. 2011, Sersa et al. 2012, Testori et al. 2012, Gargiulo et al. 2012, Kis et al. 2012]

Zdravljenje kožnih in podkožnih tumorjev z ECT rutinsko uporabljajo v klinični praksi v vedno večjem številu medicinskih ustanov po svetu [Magjarevic et al. 2011], predvsem odkar je na tržišču dostopna medicinska naprava za ECT – Cliniporator (IGEA, Carpi, Italija) [Bertacchini et al. 2007]. Uporaba ECT v kliniki narašča tudi zaradi njenih ugodnih karakteristik: visoka učinkovitost, varnost, enostavnost, nizka toksičnost, možnost ambulantne uporabe, nizki stroški in možnost večkratnega ponavljanja terapije [Marty et al. 2006, Sersa et al. 2008b, Snoj et al. 2005, Snoj et al. 2009, Colombo et al. 2008, Moller et al. 2009, Quaglino et al. 2008, Magjarevic et al. 2008, Campana et al. 2009, Campana et al. 2010, Testori et al. 2009, Testori et al. 2010]. V zadnjem času za namene zdravljenja globoko ležečih tumorjev razvijajo nove postopke zdravljenja, ki vključujejo kirurške, prekokožne in endoskopske pristope za dostop do področja zdravljenja [Soden et al. 2006, Miklavcic et al. 2010, Magjarevic et al. 2011, Edhemovic et al. 2011, Agerholm-Larsen et al. 2011, Mahmood & Gehl 2011, Linnert et al. 2012].

VARNOST ELEKTROKEMOTERAPIJE

V vseh kliničnih člankih o uporabi ECT za zdravljenje kožnih in podkožnih tumorjev poročajo, da je metoda varna za pacienta in ne povzroča resnih stranskih učinkov, ki bi bili posledica ECT [Marty et al. 2006]. Znani so le manjši stranski učinki ECT, izzvani zaradi dovajanja elektroporacijskih pulzov in/ali injiciranja kemoterapevtika [Domenge et al. 1996, Heller et al. 1998, Shimizu et al. 2003, Bloom & Goldfarb 2005, Campana et al. 2009]. Dovajanje elektroporacijskih pulzov lahko pri pacientih povzroči manjše, lokalizirane in začasne poškodbe na normalnem tkivu, ki je med terapijo v neposrednem stiku z elektrodami, in akutno lokalizirano bolečino, povezano s kontrakcijo skeletnih mišic v bližini elektrod [Marty et al. 2006, Zupanic et al. 2007]. Če pa bi z električnimi pulzi sprožili kontrakcijo srčne mišice, bi to lahko predstavljalo resen problem [Reilly 1998]. Pri trenutnem protokolu dovajanja elektroporacijskih pulzov za zdravljenje kožnih in podkožnih tumorjev obstaja zelo majhna možnost, da bi lahko vplivali na delovanje srca, saj so pulzi kratkega trajanja in so dovedeni relativno daleč od srca (glede na majhno razdaljo med elektrodami). Manjše hemodinamične ali kardiološke spremembe med ECT so opazili le v nekaj študijah, izražene pa so bile kot srčna aritmija, padec v osnovnem nivoju elektrokardiograma (EKG) ali začasna pospešena frekvenca bitja srca s povečanim maksimalnim krvnim tlakom [Domenge et al. 1996, Shimizu et al. 2003, Bloom & Goldfarb 2005]. Ker pa do danes še nihče ni sistematično preučil možnosti vpliva elektroporacijskih pulzov na delovanje srca, ne moremo trditi, da so te spremembe nujno nastale v povezavi z ECT.

Razmere glede varnosti s stališča delovanja srca pa so se nedavno bistveno spremenile z uporabo ECT za zdravljenje notranjih, globoko ležečih tumorjev, do katerih dostopajo bodisi kirurško, prekokožno (z dolgimi "globokimi" elektrodami) ali endoskopsko zdravljenja [Soden et al. 2006, Miklavcic et al. 2010, Magjarevic et al. 2011, Edhemovic et al. 2011, Agerholm-Larsen et al. 2011, Mahmood & Gehl 2011, Linnert et al. 2012]. Področje zdravljenja se namreč lahko v takih razmerah nahaja relativno blizu srca. Poleg tega pa se električni tok, ki ga dovedemo med ECT, zaradi odsotnosti sloja kože in drugih podkožnih tkiv, ki služijo kot zaščitna pregrada, in relativno velike električne prevodnosti notranjih tkiv in organov, lahko razširi po večji prostornini tkiva v okolici področja zdravljenja. V takih razmerah obstaja večja verjetnost za vpliv elektroporacijskih pulzov na delovanje srca in s tem za morebitne škodljive učinke. V nedavno objavljenih kliničnih študijah o netermični

ireverzibilni elektroporaciji globoko ležečih tumorjev (na jetrih, pljučih, ledvicah in srcu), kjer se uporablja praktično enake elektroporacijske pulze kot pri ECT, poročajo o različnih hemodinamičnih in kardioloških spremembah zaradi dovajanja elektroporacijskih pulzov, in sicer o povišanju sistoličnega tlaka, supraventrikularni tahikardiji, ventrikularni tahikardiji s padcem tlaka, ventrikularni fibrilaciji, dvigu nivoja ST segmenta in spremembah v valu T [Ball et al. 2010, Thomson 2010, Deodhar et al. 2011].

Med možnimi nepravilnostmi v delovanju srca, ki bi jih lahko povzročilo dovajanje elektroporacijskih pulzov, je za življenje pacienta najbolj nevarna ventrikularna fibrilacija [Reilly 1998]. V splošnem fibrilacijo lahko izzovemo, če dovedeni električni tok v nekem delu srca preseže pragovno vrednost za fibrilacijo [Reilly 1998]. Pragovna vrednost za fibrilacijo se pomembno zniža, če električni dražljaj dovedemo med t.i. dobo občutljivosti za atrije in ventrikle [Wiggers & Wegria 1940, Jones & Geddes 1977, Reilly 1998]. Doba občutljivosti za ventrikle sovpada s področjem vala T, doba občutljivosti za atrije pa se nahaja nekje na valu S v signalu EKG [Kirchhof et al. 1996, Reilly 1998, Ayers et al. 1994]. Verjetnost, da bi površinski električni pulzi dovedeni izven dobe občutljivosti lahko sprožili ventrikularno fibrilacijo, je izredno majhna [Reilly 1998]. Verjetnost, da elektroporacijski pulzi vplivajo na delovanje srca, je odvisna tudi od uporabljene napetosti, trajanja, števila in ponavljalne frekvence elektroporacijskih pulzov ter od poti električnega toka [Reilly 1998]. Poleg tega se prag za fibrilacijo zaradi pojava nekaterih aritmij (npr. ob pojavu prezgodnjih utripov) lahko začasno zniža za do 35%, zato je srce takrat bolj dovzetno za zunanje dražljaje in lažje nehote izzovemo fibrilacijo [Reilly 1998].

Na delovanje srca lahko vpliva tudi kemoterapevtik [Loerzel & Dow 2003, Yeh et al. 2004, Curigliano et al. 2010]. Škodljivi vplivi bleomicina in cisplatina, ki se ju uporablja v ECT, se lahko odražajo s pojavom ali povečanim številom pojavov prezgodnjih atrijskih utripov, s pojavom atrijske tahikardije, bradikardije ali sprememb v prevajanju [Tomirotti et al. 1984, Allen 1992, Villani et al. 1994, Tassinari et al. 1997, Bloom & Goldfarb 2005, Nuver et al. 2005, Yavas et al. 2008].

Dovajanju elektroporacijskih pulzov med dobo občutljivosti in ob pojavu srčnih aritmij se je priporočljivo izogibati. To lahko dosežemo tako, da dovajanje elektroporacijskih pulzov sinhroniziramo z valom R v signalu EKG, ki sovpada z najvarnejšo dobo za dovajanje pulzov [Bertacchini et al. 2007, Bertacchini et al. 2010, Ball et al. 2010, Deodhar et al. 2011].

Medicinska naprava za ECT kožnih in podkožnih tumorjev (Cliniporator, IGEA, Carpi, Italy) možnosti sinhronizacije ni vključevala, nova generacija medicinske naprave za ECT globoko ležečih tumorjev (Cliniporator Vitae) pa tako sinhronizacijo omogoča, vendar z določenimi pomanjkljivostmi [Bertacchini et al. 2010]. Zanesljiva sinhronizacija dovajanja elektroporacijskih pulzov z EKG je nujno potrebna za večjo varnost pacienta med zdravljenjem z ECT.

Učinkovitost elektrokemoterapije

Za namene naše študije učinkovitosti ECT smo opazovali odziv na terapijo na ravni tumorja, saj je ECT terapija za lokalno zdravljenje tumorjev. Klinično gledano pa je sicer pomembnejši odziv na terapijo na ravni pacienta. Odziv posameznega tumorja lahko razvrstimo v eno izmed štirih kategorij: popolni odziv (CR – angl. complete response), delni odziv (PR – angl. partial response), mirovanje (NC – angl. no change) ali napredovanje bolezni (PD – angl. progressive disease) glede na kriterije WHO in RECIST [World Health Organization 1979, Therasse et al. 2000]. V kliničnih študijah poročajo o deležu popolnih (CR) in objektivnih (OR, ki je vsota CR in PR) odzivov (z oznako CR% in OR%), ki predstavljata skupen odziv za vse tumorje vključene v študijo.

ECT je učinkovito zdravljenje s CR% med 60 in 70% ter OR% okrog 80% [Sersa et al. 2008c, Marty et al. 2006, Sersa 2006, Landstrom et al. 2010, Kis et al. 2011, Matthiessen et al. 2011]. Nekaj preglednih člankov, ki povzemajo podatke o učinkovitosti ECT, je sicer bilo objavljenih [Mir et al. 1998, Goldfarb et al. 2005, Marty et al. 2006, Larkin et al. 2007, Moller et al. 2009], a sistematičnega pregleda učinkovitosti klinične ECT do danes še ni bilo objavljenega. Sistematičen pregled, ki bi temeljil na statističnem združevanju podatkov iz različnih študij objavljenih do danes, je potreben, saj bi dal jasno in objektivno osnovo za diskusijo o tako imenovanem skupnem učinku zdravljenja z ECT [Borenstein et al. 2009].

Znotraj projekta ESOPE (angl. European Standard Operating Procedures of Electrochemotherapy) so bili za namene uporabe ECT pripravljeni standardni operativni postopki (SOP – angl. standard operating procedures) za varno in učinkovito zdravljenje, ki temeljijo na izkušnjah vodilnih evropskih centrov za zdravljenje raka z ECT [Mir et al. 2006]. SOP vsebujejo odločitveno drevo, ki pomaga zdravniku pri odločanju med različnimi možnimi načini zdravljenja glede na število kožnih tumorjev ter njihovo lokacijo in premer največjega tumorja.

Kljub upoštevanju SOP pa se v učinkovitosti ECT med posameznimi študijami kaže velika variabilnost, kar lahko pripišemo predvsem različnim pogojem zdravljenja, pod katerimi je bila ECT izvedena. Za učinkovito ECT je v prvi meri potrebno, da zagotovimo zadostno koncentracijo kemoterapevtika v tumorju v času dovajanja elektroporacijskih pulzov. Hkrati moramo na tumor dovesti zadosti visoko električno polje, ki bo povzročilo elektroporacijo membrane celic v celotnem tumorju in tako omogočilo vnos kemoterapevtika v celice [Domenge et al. 1996, Miklavcic et al. 1998, Miklavcic et al. 2000, Miklavcic et al. 2006]. Poleg tega na učinkovitost ECT vplivajo tudi pogoji zdravljenja povezani s pacientom, tumorjem ali postopkom zdravljenja (na primer starost; spol; histološki izvor, lokacija in velikost tumorja; vrsta, doza in način injiciranja kemoterapevtika; vrsta uporabljenih elektrod; vrednost dovedenega toka, napetosti in energije na volumen tumorja; protokol in čas dovajanja elektroporacijskih pulzov; čas opazovanja odziva tumorja). Vpliv pogojev zdravljenja na učinkovitost ECT ni ustrezno raziskan, ali pa študije kažejo med seboj kontradiktorne rezultate [Rodriguez-Cuevas et al. 2001, Rebersek et al. 2004, Marty et al. 2006, Larkin et al. 2007, Quaglino et al. 2008, Campana et al. 2009, Campana et al. 2012], zato bi bilo njihovo vlogo potrebno preučiti. Razumevanje njihove vloge bi lahko služilo nadaljnji optimizaciji postopkov klinične ECT, kar bi privedlo do najboljšega možnega rezultata zdravljenja z ECT.

Cilji

Disertacija ima tri glavne cilje. Prvi cilj je preučiti varnostne vidike ECT, kar vključuje iskanje možnih učinkov elektroporacijskih pulzov in kemoterapevtika na delovanje srca. Drugi cilj disertacije je razvoj algoritma za učinkovito in zanesljivo sinhronizacijo dovajanja elektroporacijskih pulzov z EKG. Tretji cilj disertacije pa je sistematičen pregled objavljenih člankov o klinični ECT in določitev skupne učinkovitosti ECT ter ovrednotenje učinkov različnih vplivnih pogojev na učinkovitost ECT.

Materiali in metode

Varnost elektrokemoterapije

Da bi lahko ovrednotili varnost ECT, smo zgradili različne matematične modele kožnega tumorja v mišičnem tkivu z enako geometrijo in obliko elektrod, ki se uporabljajo v klinični ECT. Modelirali smo tri različne tipe elektrod: ploščate, igelne z geometrijo v dveh

vrstah in igelne s heksagonalno geometrijo. Numerične izračune električnega polja in tokovne razporeditve smo izvedli z metodo končnih elementov programskega paketa COMSOL Multiphysics 3.3 (COMSOL AB, Sweden) za različne razdalje med ploščatimi elektrodami in za različne globine vstavljenih igelnih elektrod. Določili smo teoretične pogoje (vrsta uporabljenih elektrod, globina vstavitve), ko bi lahko z elektroporacijskimi pulzi vplivali na delovanje srca [**Paper I**].

Varnostni vidik ECT smo ovrednotili tudi na signalih EKG, zajetih med klinično uporabo ECT na Onkološkem inštitutu Ljubljana, kjer smo ugotavljali spremembe v EKG, ki bi lahko nastale zaradi injiciranja kemoterapevtika in/ali dovajanja elektroporacijskih pulzov. V ta namen smo uporabili dva različna sistema za zajem signala EKG. Za ugotavljanje zgodnjih učinkov ECT (t.j. učinkov, ki se pojavijo med ali takoj za zdravljenjem z ECT) na delovanje srca smo uporabili relativno kratke (približno dve-urne) posnetke signala EKG med ECT kožnih in podkožnih tumorjev, kjer se področje zdravljenja nahaja na koži in tako leži relativno daleč od srca [**Paper I**], ter med ECT globoko ležečih tumorjev (kolorektalnih metastaz na jetrih), kjer se področje zdravljenja nahaja relativno blizu srca [**Paper II**, **Paper IV**]. Za ugotavljanje zakasnelih učinkov ECT (t.j. učinkov, ki se pojavijo v 24-ih urah po zdravljenju z ECT) na delovanje srca pa smo uporabili daljše (približno 24-urne) signale EKG zajete pred in po ECT globoko ležečih tumorjev [**Paper III**]. Razvili smo algoritem za natančno analizo zajetih signalov EKG [**Paper I**, **Paper V**]. Algoritem svoje parametre prilagodi karakteristikam analiziranega signala EKG in določi pomembne lastnosti posameznega srčnega utripa (npr. izoelektrični nivo, amplituda vala R, trajanje intervalov RR in QT) ter lastnosti signala EKG preko več srčnih utripov z različnimi pristopi (npr. povprečja različnih intervalov srčnih utripov, kot sta interval RR in popravljeni interval QT preko različnih časovnih intervalov; analiza variabilnosti srčnega ritma; uporaba klasične statistične analize). Rezultate analize smo uporabili za kvantitativno ovrednotenje sprememb na EKG med in po ECT. Določili smo še, ali so morebitne spremembe v signalu EKG povezane z uporabljenimi električnimi parametri med ECT [**Paper II**, **Paper III**].

Algoritem za sinhronizacijo dovajanja elektroporacijskih pulzov z elektrokardiogramom

Algoritem za sinhronizacijo dovajanja elektroporacijskih pulzov z EKG sestavljajo trije podsklopi: za fazo učenja; za zaznavanje kompleksov QRS; ter za klasifikacijo srčnih utripov in odločanje o dovajanju elektroporacijskih pulzov (glej sliko 6 v Materialih in metodah disertacije) [**Paper V**]. Algoritem za sinhronizacijo elektroporacijskih pulzov z EKG zadošča vsem štirim potrebnim pogojem, ki morajo biti izpolnjeni za možno uporabo v ECT. Prvič, algoritem prilagodi svoje parametre lastnostim analiziranega signala EKG, kar vsebuje podsklop za fazo učenja. Drugič, sklop za zaznavanje kompleksov QRS omogoča zgodnje zaznavanje kompleksa QRS, in sicer na veznici QR, in temelji na preprostih metodah (prvi in drugi odvod signala, amplitude valov srčnega utripa in vrednost intervala RR), kar omogoča delovanje v realnem času. Tretjič, algoritem iz srčnega utripa izlušči lastnosti, ki omogočajo dobro razlikovanje med normalnimi srčnimi utripi in aritmijami (npr. amplituda vala R, interval RR, povprečne vrednosti teh lastnosti in odstopanja lastnosti posameznega srčnega utripa od povprečnih vrednosti). Četrtič, faza odločanja algoritma glede sinhroniziranega dovajanja elektroporacijskih pulzov z EKG omogoča dovajanje izven dobe občutljivosti in ob odsotnosti srčnih aritmij. Delovanje algoritma smo najprej ovrednotili na signalih EKG iz standardne baze Long-term ST z označenimi in klasificiranimi srčnimi utripi [**Paper V**], za tem pa na signalih EKG zajetih med ECT [**Paper I**]. Delovanje algoritma smo primerjali z obstoječim protokolom sinhronizacije, ki je vključen v klinično napravo za ECT, in glede na rezultate primerjave predlagali izboljšavo obstoječega protokola sinhronizacije [**Paper II**].

Učinkovitost elektrokemoterapije

Za namene ovrednotenja učinkovitosti ECT smo izvedli sistematičen pregled rezultatov študij o klinični ECT [**Paper VI**, **Paper VII**]. V različnih bibliografskih podatkovnih bazah smo poiskali vse objavljene članke, ki zadevajo uporabo klinične ECT in so primerni za statistično združevanje podatkov. Iz člankov smo, kjer je bilo mogoče, razbrali surove podatke o parametrih zdravljenja (npr. podatke o pacientu, lastnostih tumorja in uporabljenih električnih parametrih elektrokemoterapije) ter odzivu tumorja. Poleg tega smo identificirali tudi dve ustrezni podatkovni bazi o ECT kožnih in podkožnih tumorjev (baza Onkološkega inštituta Ljubljana in baza Onkološkega inštituta iz Padove v Italiji) [**Paper**

VII]. Podatke o odzivih tumorjev smo sistematično analizirali s klasičnimi statističnimi pristopi (npr. Chi-square test, t test) in z meta-analizo [**Paper VI**, **Paper VII**]. Na ta način smo lahko določili skupno učinkovitost ECT kot tudi vplivne parametre, ki vplivajo na rezultate zdravljenja z ECT, in razložili njihov učinek na učinkovitost ECT [**Paper VI**, **Paper VII**].

REZULTATI IN ZAKLJUČKI

VARNOST ELEKTROKEMOTERAPIJE

Rezultati numeričnih izračunov matematičnega modela kožnega tumorja nakazujejo teoretično možnost, da v pogojih, ko uporabljamo igelne elektrode vstavljene 1 cm v globino na prsnem košu tik nad srcem, izzovemo fibrilacijo srca. Kritična globina, ko bi teoretično lahko izzvali fibrilacijo srca, je bila namreč ocenjena na okrog 4 cm.

Rezultati analize signalov EKG zajetih med ECT kožnih, podkožnih in globoko ležečih tumorjev kažejo, da ECT ne povzroča nobenih resnih učinkov na delovanje srca [**Paper I**, **Paper II**, **Paper III**, **Paper IV**]. Ugotovili pa smo, da ECT povzroča zgodnje učinke, ki se odražajo kot začasno skrajšanje intervalov RR in QRS, oziroma povišanje frekvence bitja srca, kadar so elektroporacijski pulzi dovedeni na kožne in podkožne tumorje brez sinhronizacije in v lokalni anesteziji [**Paper I**]. Te začasne spremembe lahko večinoma, če ne v celoti, pripišemo strahu in stresu pacienta, ki prestaja ECT. Tudi rezultati analize signalov EKG posnetih med ECT tumorjev na jetrih kažejo zgodnje učinke na delovanje srca, čeprav je bilo dovajanje elektroporacijskih pulzov sinhronizirano z EKG [**Paper II**, **Paper IV**]. Učinki so se izražali kot začasno skrajšanje korigiranega intervala QT in povečanje kratkotrajne variabilnosti srčnega ritma. Pojavili so se najverjetneje zaradi električne stimulacije mišic in živcev v bližnji okolici dovajanja elektroporacijskih pulzov, za katero je znano, da posledično izzove kardiovaskularni odziv, ki se izraža z začasnim dvigom frekvence srčnega ritma. Našli smo statistično značilno korelacijo med spremembami korigiranega QT intervala in tokom ter energijo dovedeno med ECT. Statistično značilno korelacijo je zaznati tudi med spremembami intervala RR in vrednostjo toka med terapijo.

Rezultati analize signalov EKG posnetih pred in po ECT tumorjev na jetrih nakazujejo, da obstajajo tudi zakasnjeni učinki ECT na delovanje srca, ki se izražajo v obliki povišane frekvence bitja srca oziroma skrajšanja intervala RR in v obliki zmanjšanja parametrov dolgotrajne variabilnosti srčnega ritma (Poincaréjevega deskriptorja SD2 in nizko-frekvenčne

komponente LF) [**Paper III**]. Te spremembe gre najverjetneje vsaj delno pripisati učinkom analgetikov in drugih zdravil, ki jih pacienti prejmejo v intenzivni oskrbi, in pooperativni bolečini. Pokazali smo namreč tudi, da so te spremembe statistično značilno negativno korelirane s številom dovedenih elektroporacijskih pulzov na pacienta.

Za zaključek lahko trdimo, da z ECT sicer lahko vplivamo na delovanje srca, vendar bodo ti učinki najverjetneje začasni in brez resnih zapletov, ki bi ogrožali življenje pacienta. Vseeno pa bi se verjetnost za škodljive stranske učinke lahko povečala ob morebitni uporabi elektroporacijskih pulzov z daljšim trajanjem ali večjim številom pulzov z zvišano ponavljalno frekvenco in/ali pri zdravljenju tumorjev v neposredni bližini srca. V objavi naše študije na to temo [**Paper I**] smo prvi priporočili, da bi moralo biti dovajanje elektroporacijskih pulzov sinhronizirano z absolutno refraktorno dobo srčnega cikla (to je z valom R), da pacientu zagotovimo največjo možno varnost. Danes se sinhronizacija elektroporacijskih pulzov rutinsko uporablja pri zdravljenju globoko ležečih tumorjev z ECT in s postopki ireverzibilne elektroporacije.

Algoritem za sinhronizacijo dovajanja elektroporacijskih pulzov z elektrokardiogramom

Razvili smo zanesljiv algoritem za sinhronizacijo dovajanja elektroporacijskih pulzov z EKG [**Paper V**]. Vrednotenje algoritma na signalih EKG iz standardne baze Long-term ST in na signalih zajetih med klinično ECT dokazuje, da razviti algoritem omogoča varnejšo ECT z vidika pacienta, saj dovaja elektroporacijske pulze le izven trenutkov, ki so lahko za pacienta nevarni [**Paper I**, **Paper V**]. Algoritem namreč dovaja elektroporacijske pulze le izven dobe občutljivosti ventriklov in ob odsotnosti srčnih aritmij, kot so atrijski in ventrikularni prezgodnji utripi. Algoritem omogoča izvedbo v realnem času, še več, ocenjena časovna rezerva za še vedno varno dovajanje elektroporacijskih pulzov znaša okrog 60 ms [**Paper I**, **Paper V**]. Algoritem predstavlja pomembno izboljšavo v primerjavi z obstoječim protokolom za sinhronizirano dovajanje elektroporacijskih pulzov vgrajenim v klinično napravo za ECT, še posebej v primeru zdravljenja globoko ležečih tumorjev v bližini srca [**Paper II**]. Obstoječi protokol sinhronizacije je nujno čim preje izboljšati ali nadomestiti z našim algoritmom, predvsem zaradi pričakovanega porasta uporabe ECT za zdravljenje globoko ležečih tumorjev, kjer se področje zdravljenja lahko nahaja relativno blizu srca.

Zaključimo lahko, da je razviti algoritem za sinhronizacijo dovajanja elektroporacijskih pulzov z EKG zaradi učinkovitega in zanesljivega delovanja razen za ECT primeren tudi za uporabo v vseh ostalih medicinskih aplikacijah, ki vključujejo dovajanje visokonapetostnih pulzov kot na primer pri genski elektrotransfekciji in raznih uporabah ireverzibilne elektroporacije. V prihodnje bi lahko razviti algoritem nadgradili, da bi zdravljenje s temi aplikacijami omogočal tudi trenutno za to terapijo neustreznim pacientom; to je pacientom s klinično izraženimi aritmijami in vgrajenim srčnim spodbujevalnikom.

Učinkovitost elektrokemoterapije

Da bi povzeli trenutno znanje in izkušnje na področju zdravljenja z ECT z vidika učinkovitosti, smo izvedli sistematičen pregled literature na temo klinične ECT [**Paper VI**, **Paper VII**]. Za ECT izvedeno le enkrat na posameznem kožnem ali podkožnem tumorju in ne glede na različne izhodiščne pogoje in uporabljene parametre smo ocenili skupno učinkovitost. Delež popolnih odzivov CR% znaša 59.4%, delež objektivnih odzivov OR% pa 84.1% [**Paper VI**]. Rezultati kažejo, da elektroporacijski pulzi statistično značilno povečajo učinkovitost kemoterapevtika za več kot 50% [**Paper VI**]. Pokazali smo, da so spremembe v učinkovitosti ECT statistično značilno odvisne vsaj od treh izhodiščnih pogojev zdravljenja, in sicer od vrste kemoterapevtika, histološkega izvora tumorja in velikosti tumorja [**Paper VI**, **Paper VII**]. Pokazali smo, da je učinkovitost ECT na kožnih in podkožnih tumorjih statistično značilno višja za intratumorsko kot intravensko dovajanje bleomicina. Injiciranje bleomicina ali cisplatina intratumorsko rezultira v enaki učinkovitosti ECT. Tumorji, ki niso melanomskega izvora, se odzivajo na zdravljenje z ECT statistično značilno bolje kot melanomi. Izmed vseh histoloških tipov tumorjev se bazalno-celični karcinomi najbolje, ploščato-celični karcinomi pa najslabše odzivajo na zdravljenje z ECT. Rezultati analize podatkov iz dveh baz o ECT kožnih in podkožnih tumorjev dokazujejo, da je učinkovitost ECT na tumorjih večjih od 3 cm statistično signifikantno nižja kot na manjših tumorjih in upada progresivno z naraščajočo velikostjo tumorja.

Rezultati sistematičnega pregleda literature v naši študiji pomembno prispevajo k boljšemu razumevanju vpliva nekaterih izhodiščnih pogojev in parametrov zdravljenja z ECT na končno učinkovitost te terapije. Te rezultate bi bilo smiselno upoštevati pri izboljšanju standardnih operativnih postopkov za kožne in podkožne tumorje kot tudi pri razvoju različnih postopkov za zdravljenje globoko ležečih tumorjev.

1. INTRODUCTION

It has been known since 1943 that high-voltage electroporation pulses can affect functioning of a cell, when Goldman published the first report about abrupt increase of conductivity of the cell membrane and consequently electrical breakdown of the cell membrane [Goldman 1943]. Later, Stampfli revealed that this electrical breakdown of the membrane can be reversible [Stampfli 1958]. Ten years later, two researchers initiated their studies on non-thermal killing of microorganisms by means of high electric fields [Sale & Hamilton 1967, Hamilton & Sale 1967, Sale & Hamilton 1968]. Soon afterwards, Neuman and Rosenheck reported on reversible membrane permeability changes induced by electric pulses and proposed this effect could be used for drug transfer into the cells [Neumann & Rosenheck 1972]. The first practical evidence for gene transfer *in vitro* using electric pulses followed in 1982, when Neuman et al. also introduced the term 'electroporation' as expression for permeabilization of the cell membrane with electrical fields [Neumann et al. 1982]. This study was a milestone that opened new possibilities for future exploration. One of the greatest breakthroughs in the field of electroporation was successful use of electroporation in combination with anticancer drugs for treatment of *in vivo* growing tumors on rats by Okino and Mohri in 1987 [Okino & Mohri 1987]. Independently, in 1988, Orlowski et al. published systematic study *in vitro*, in which they proposed the use of electroporation to reversibly permeabilize cells and thereby introduce more effectively cytotoxic agents into malignant cells. This field has subsequently developed to become an important application of reversible electroporation for treatment of tumors. In 1991, Mir et al. proved that this promising treatment of tumors, termed as 'electrochemotherapy', is also feasible on humans and, furthermore, reported their impressive results [Mir et al. 1991a, Belehradek et al. 1993].

1.1. ELECTROPORATION

If a cell, a cluster of cells or tissue is exposed to an electric field, an induced voltage difference across the cell membrane is created, which is superimposed to the resting membrane potential [Kotnik et al. 1997, Teissie et al. 1999, Miklavcic et al. 2000]. When the total transmembrane potential difference exceeds a threshold value (ranging between 200 mV and 1 V) [Neumann et al. 1982, Weaver & Chizmadzhev 1996, Weaver 2000, Kotnik et al.

2010], a rearrangement of the molecular structure of the membrane occurs. This leads to the formation of hydrophilic pores in the membrane and to a significant increase in the cell membrane permeability [Weaver & Chizmadzhev 1996, Kotnik et al. 1997, Weaver 2000, Chen et al. 2006]. This phenomenon is termed electroporation or electropermeabilization. Increased membrane permeability allows molecules, which under physiological conditions cannot cross the cell membrane, to enter the cell (Figure 1) [Mir et al. 1991b]. Electroporation can be reversible or irreversible, depending on electrical conditions (number, shape, amplitude, duration and repetition frequency of electric pulses, and direction of electric field) and cell or tissue characteristics [Macek Lebar et al. 2002, Zupanic et al. 2008, Cemazar et al. 2009, Miklavcic & Towhidi 2010, Miklavcic et al. 2010]. Parameters for reversible electroporation are selected in a way that the cell after application of electroporation pulses is able to reestablish homeostasis, so that the viability of the electroporated cell is preserved. On the other hand, irreversible electroporation cause permanent permeabilization of the cell and consequent cell death.

Both reversible and irreversible electroporation have important applications in biotechnology and medicine [Mir 2000, Dev et al. 2000]. Reversible electroporation is now used in electrochemotherapy [Gehl & Geertsen 2006, Marty et al. 2006, Larkin et al. 2007, Sersa et al. 2008c, Moller et al. 2009, Campana et al. 2010, Testori et al. 2010, Richetta et al. 2011, Hampton 2011, Kis et al. 2011, Edhemovic et al. 2011, Curatolo et al. 2012, Sersa et al. 2012, Campana et al. 2012, Escoffre & Rols 2012], gene electrotransfection [Andre & Mir 2004, Andre et al. 2008, Pavlin et al. 2008, Gothelf & Gehl 2010, Littel-van den Hurk & Hannaman 2010, Heller & Heller 2010, Hojman 2010], transdermal drug delivery [Prausnitz 1999, Denet et al. 2004, Pavselj & Preat 2005, Kalluri & Banga 2011, Wong et al. 2011], and cell electrofusion [Mekid & Mir 2000, Trontelj et al. 2008, Salomskaite-Davalgiene et al. 2009, Usaj et al. 2010], whereas irreversible electroporation is used for food processing [Qin et al. 1995, Jayaram 2000, Vernhes et al. 2002, Golberg et al. 2010] and tissue ablation [Davalos et al. 2005, Rubinsky 2007, Maor et al. 2009, Pech et al. 2011, Tracy et al. 2011].

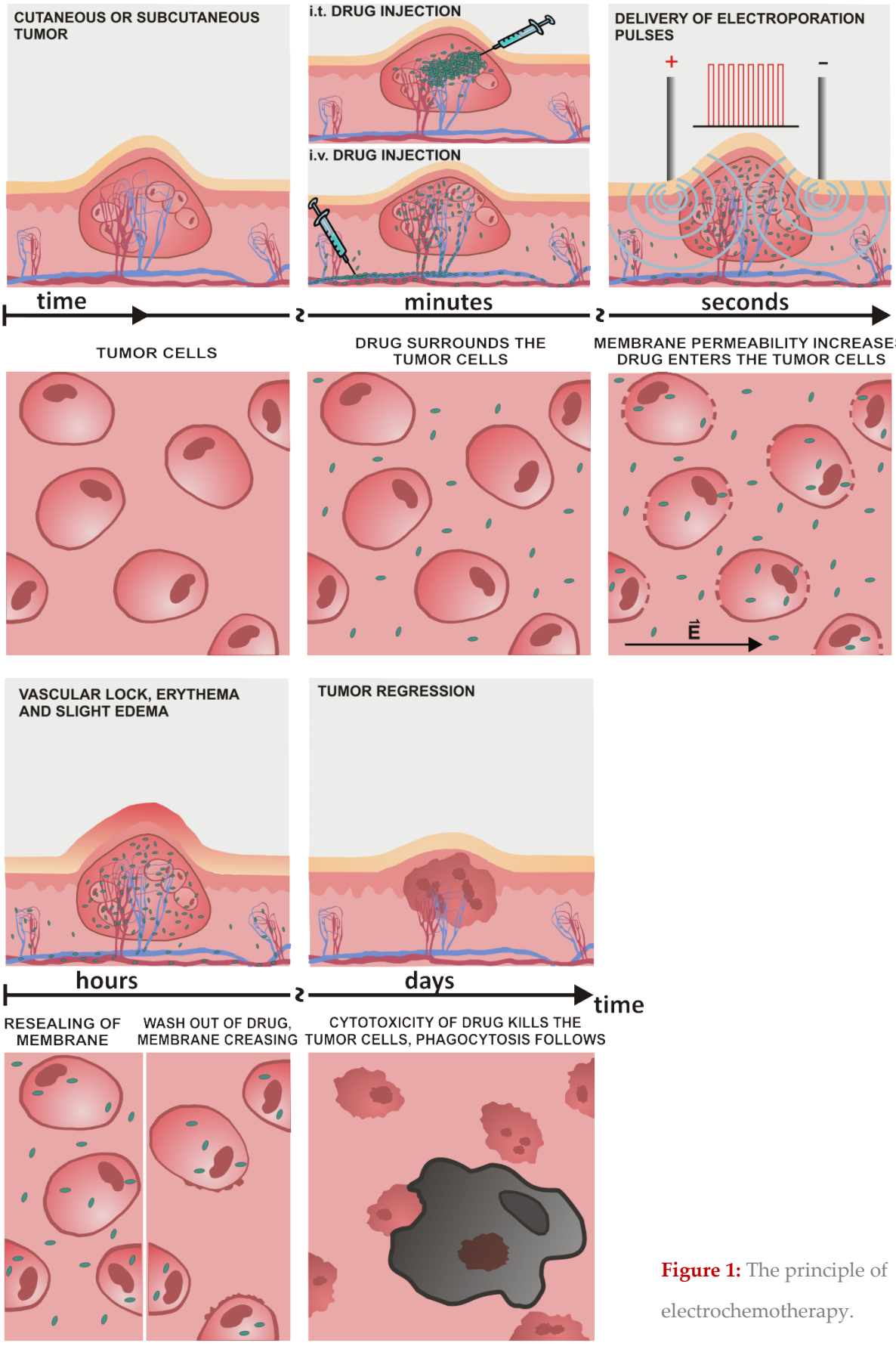

Figure 1: The principle of electrochemotherapy.

1.2. ELECTROCHEMOTHERAPY

Electrochemotherapy (ECT) is a treatment of tumors in which either systemic or local injection of a cytotoxic chemotherapeutic drug is followed by application of short high-voltage electroporation pulses locally to the tumor. Electroporation pulses transiently increase permeability of the cell membrane, and thus enable nonpermeant or poorly permeant antitumor drug (such as bleomycin and cisplatin) to cross the plasma membrane of tumor cells and to exert its cytotoxic effect (Figure 1). The chemotherapeutic drug alone or electroporation pulses alone have minimal or no effect on tumor growth. Therefore, the antitumor effectiveness is considerably higher for ECT than for systemic chemotherapy, although much lower drug doses can be used in ECT [Mir et al. 1991b, Sersa et al. 1995]. The patients can thus receive a single-shot treatment with limited systemic toxicity, but the treatment can also be repeated, if necessary, thus producing better responses [Quaglino et al. 2008, Campana et al. 2009, Testori et al. 2010].

Other mechanisms contributing to high effectiveness of ECT were recognized besides the permeabilization mechanism of ECT: a vascular disrupting effect (the severely damaged tumor vasculature due to ECT of endothelial cells), vascular lock effect (a temporary reduction in perfusion of the tumor tissue due to electroporation pulses) and the action of patient's immune system (Figure 1) [Sersa et al. 2008a, Jarm et al. 2010]. The vascular disrupting effect leads to an additional cascade of tumor cell death as a result of long-term lack of oxygen and nutrients and accumulation of waste products in the tumor. If sufficiently high intratumoral drug concentration is present at the time of delivery of electroporation pulses, the vascular lock effect retains antitumor drug, which can thus increase its local cytotoxic activity before it is cleared from tumor tissue. The vascular disrupting and vascular lock effects have been successfully exploited by using ECT in the treatment of bleeding melanomas [Gehl & Geertsen 2000, Gehl & Geertsen 2006, Snoj et al. 2009]. The action of patient's immune system is an additional mechanism playing an important complementary role in attaining high effectiveness of ECT [Sersa et al. 1997, Daud et al. 2008, Cemazar et al. 2010, Jarm et al. 2010].

Since the first clinical study in 1990 [Mir et al. 1991a, Belehradek et al. 1993], ECT has been reported as a highly effective treatment [Marty et al. 2006, Sersa 2006, Sersa et al. 2008b,

Landstrom et al. 2010, Kis et al. 2011, Matthiessen et al. 2011, Hampton 2011, Sersa et al. 2012, Campana et al. 2012, Escoffre & Rols 2012]. In 2006, standard operating procedures (SOP) for ECT using Cliniporator device were prepared, based on the experience from the leading European cancer centers using ECT [Mir et al. 2006]. The aim of the SOP document was to define guidelines for safe and effective ECT of cutaneous and subcutaneous tumors with respect to number, size (maximal diameter) and depth of tumors. Treatment of cutaneous and subcutaneous tumors using ECT is routinely used in everyday clinical practice and its use is increasing [Magjarevic et al. 2011]. The reason for an increasing use of ECT in clinics arises from favorable treatment characteristics, which are: high effectiveness, safety, simplicity, organ sparing effect, low toxicity, possible application in an out-patient setup, cost-effectiveness, and suitability for repetitive and neoadjuvant treatment therapy [Marty et al. 2006, Sersa et al. 2008b, Snoj et al. 2005, Snoj et al. 2009, Colombo et al. 2008, Moller et al. 2009, Quaglino et al. 2008, Magjarevic et al. 2008, Campana et al. 2009, Campana et al. 2010, Testori et al. 2009, Testori et al. 2010]. ECT has already been approved and is covered by medical insurance in several EU countries, including Slovenia. Recently, new ECT procedures are being developed for treatment of deep seated tumors by means of ECT using surgical procedures, endoscopic routes or percutaneous approaches to gain access to the treatment area [Soden et al. 2006, Miklavcic et al. 2010, Magjarevic et al. 2011, Edhemovic et al. 2011, Agerholm-Larsen et al. 2011, Mahmood & Gehl 2011, Linnert et al. 2012].

A typical ECT protocol involves delivery of chemotherapeutic drug (bleomycin or cisplatin) that is followed by sequential delivery of eight electroporation pulses with 100 μs duration and repetition frequency of 1 Hz or 5 kHz between each pair of the electrodes [Marty et al. 2006]. Electroporation pulses can be applied to the tumor by plate electrodes on the skin surface, or by needle electrodes inserted into the tumor. Plate electrodes are more suitable for small and superficial tumors, whereas needle electrodes are more convenient for larger and deeper seated tumors [Miklavcic et al. 2006]. Usually, voltage over distance ratio of 1300 V/cm for plate and 1000 V/cm for needle electrodes is used [Mir et al. 2006, Marty et al. 2006, Sersa et al. 2008c]. However, the voltage over distance ratio is not the actual physical parameter that determines successful outcome of therapy. The characteristics of delivered electroporation pulses depend on type and configuration of electrodes, and must be adjusted

so that threshold level for reversible electroporation of all tumor cells in treated area is exceeded [Miklavcic et al. 1998, Miklavcic et al. 2000, Miklavcic et al. 2006].

Due to relatively simple physicochemical concept of ECT which can permeabilize every type of cell, it is expected that ECT should have good antitumor effect on tumors of any histological type. Indeed, effectiveness of ECT has been demonstrated in treatment of cutaneous and subcutaneous tumors of different histological types, including malignant melanoma, head and neck squamous cell carcinoma, basal cell carcinoma, Kaposi's sarcoma and adenocarcinoma of the breast [Byrne et al. 2005, Gaudy et al. 2006, Larkin et al. 2007, Kis et al. 2011, Quaglino et al. 2008, Sersa et al. 2000, Snoj et al. 2007, Allegretti & Panje 2001, Bloom & Goldfarb 2005, Burian et al. 2003, Gargiulo et al. 2010, Landstrom et al. 2010, Sersa et al. 1998, Curatolo et al. 2008, Curatolo et al. 2012, Garbay et al. 2006, Whelan et al. 2006, Testori et al. 2011, Edhemovic et al. 2011, Sersa et al. 2012, Testori et al. 2012, Matthiessen et al. 2012].

1.3. Safety of electrochemotherapy

Advanced treatment procedures, like ECT, provide new possibilities for restoring, correcting or modifying physiological functions. At the same time, because of their novelty, complexity and technical specificity, they may bring along new, unexpected risks to patients. The first clinical studies on ECT in humans were performed in early 1990's in order to evaluate feasibility and safety [Mir et al. 1991a, Belehradek et al. 1993, Rudolf et al. 1995, Heller 1995, Domenge et al. 1996]. Due to good safety and toxicity profile, and only minor side effects, it was proved that ECT is a safe treatment [Heller et al. 1999, Larkin et al. 2007, Sersa et al. 2008c, Marty et al. 2006]. No serious early (occurring during and immediately after ECT treatment) or late effects (occurring within 24 hours after ECT treatment) related to ECT have ever been reported. Early effects are limited to minor irritation and uncomfortable sensation associated with contraction of muscles in the vicinity of the electrodes that immediately subside after delivery of each electric pulse, whereas late effects occur as slight erythema, edema and sometimes as necrosis of tissue [Mir et al. 1998, Mir & Orlowski 1999, Sersa 2006, Marty et al. 2006, Zupanic et al. 2007, Testori et al. 2011]. All these effects are local, transient, minimal and well tolerated by patients; therefore, the procedure can be applied in an out-patient setup.

Electroporation pulses stimulate nearby muscles either directly or indirectly through the nerves innervating the muscles. When tumors are located close to the heart muscle, electroporation pulses can thus potentially interfere with functioning of the heart; although none of adverse complications have been recorded so far. The likelihood of electroporation pulses influencing functioning of the heart depends on the applied voltage, duration, number and repetition frequency of electroporation pulses; inter-electrode distance; conductivity of tissue surrounding the treated region; the current pathway; and distance and location with respect to the heart [Reilly 1998]. It is highly unlikely that currently used electroporation protocols for treatment of cutaneous and subcutaneous tumors could interfere with functioning of the heart due to short pulse duration, applications mainly on locations relatively distant from the heart, and small inter-electrode distance (typically from 4 to 8 mm). However, some early minor hemodynamic or cardiologic changes during ECT performance on cutaneous and subcutaneous tumors were observed in few clinical studies, expressed as cardiac arrhythmia, a decrease in the baseline of the electrocardiogram (ECG) signal and transient heart frequency acceleration with increased maximal blood pressure [Domenge et al. 1996, Shimizu et al. 2003, Bloom & Goldfarb 2005]. Since the influence of electroporation pulses on functioning of the heart has not been systematically investigated, it is not certain if the observed hemodynamic and cardiologic changes of heart function were indeed directly related to ECT.

The safety aspect has fundamentally changed with recent development of new ECT modalities for treatment of deep seated tumors, such as tumors in bones, brain, liver, kidney colon and esophagus. The ECT techniques have changed significantly by using surgical, percutaneous or endoscopic procedures to gain access to the treatment area [Soden et al. 2006, Rubinsky 2010, Miklavcic et al. 2010, Garcia et al. 2011, Edhemovic et al. 2011, Linnert et al. 2012] that could potentially introduce new risks and side effects. In such cases the treated region can be located relatively close to the heart (e.g. in liver, lung, esophagus). In addition, due to the absence of a protective barrier of the skin, larger inter-electrode distances and/or close proximity, and relatively large electrical conductivity of internal tissues and organs, the electrical current delivered during ECT can propagate through a larger volume of tissue surrounding the treated region. There is, therefore, an increased probability of electroporation pulses affecting cardiac muscle and interfering with

functioning of the heart and thus potentially causing early or late heart-related effects. Indeed, in recently published studies on non-thermal irreversible electroporation, where practically equal electroporation pulses are used as in ECT, different minor and major hemodynamic and cardiologic changes due to unsynchronized irreversible electroporation pulse delivery were reported, such as systolic hypertension, supraventricular tachycardia, ventricular tachycardia with pressure drop, ventricular fibrillation, ST segment elevation and changes in T wave [Ball et al. 2010, Thomson 2010, Deodhar et al. 2011].

In general, there are several possible irregularities in functioning of the heart that the application of electroporation pulses could induce (e.g., atrial and ventricular flutter and fibrillation, premature heartbeats) [Reilly 1998]. The most dangerous one is ventricular fibrillation. Fibrillation can be induced if the current of the applied electric pulses in a part of the heart is greater than the threshold level for fibrillation. The heart is especially susceptible to induction of fibrillation (due to significantly lowered threshold level for fibrillation) if electrical stimulus is delivered during the late atrial or ventricular systole, during the so-called vulnerable period of the atria and ventricles, respectively (Figure 2) [Wiggers & Wegria 1940, Jones & Geddes 1977, Reilly 1998].

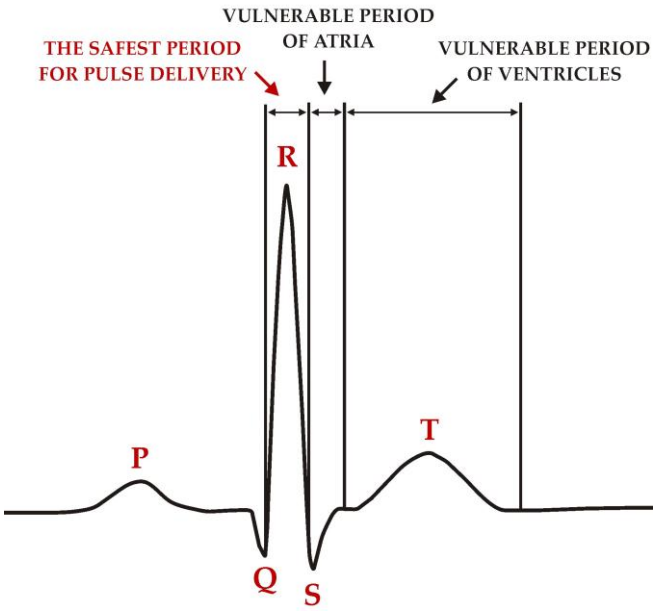

Figure 2: The vulnerable period of the atria and ventricles. During the vulnerable period, the conduction pathway is still partially refractory, so that the wave of excitation generated by stimulation can propagate in only one direction and therefore induce reentry. The atria and ventricles are not excitable during the time of QRS complex. This is the safest period in electrocardiogram for external electrical stimulation.

In the ventricles, the susceptibility to external stimulation is maximal preceding the apex of the T wave in ECG. At this time, external electrical stimulation elicits an excitation wave that encounters some regions of the heart fully recovered, other regions partially recovered, and some regions still absolutely refractory. Propagation of an electrically induced wavefront can thereby be initiated preferentially in certain directions, thus setting the stage for the so-called multiple reentry, which is the electrophysiological basis of ventricular fibrillation [Reilly 1998]. For ventricular myocardium, the vulnerable period coincides with the middle and terminal phases of the T wave [Reilly 1998], but higher-amplitude stimulus cause the vulnerable period to occur several milliseconds earlier in the heartbeat [Kirchhof et al. 1996]; therefore, the whole T wave can be considered to be within the vulnerable period of the ventricles (Figure 2). For the atria, the vulnerable period is somewhere in the S wave (Figure 2) [Ayers et al. 1994, Reilly 1998]. Externally applied electric pulses delivered outside the vulnerable period have an extremely low probability of inducing ventricular fibrillation [Reilly 1998].

Although fibrillation can occur in normal and healthy hearts, it is significantly more likely in the hearts with structural or functional abnormalities [Clayton & Holden 2000]. Some arrhythmias (i.e. abnormalities of the heart rhythm) cause the heart to become more susceptible to external stimuli due to a decreased threshold level for fibrillation. Therefore, electroporation pulses coinciding with some arrhythmias could potentially elicit fibrillation, especially after premature heartbeat, where the threshold level for fibrillation can be decreased for up to 35% [Reilly 1998].

Administration of a chemotherapeutic drug is an additional potential factor that could have an effect on functioning of the heart [Loerzel & Dow 2003, Yeh et al. 2004, Curigliano et al. 2010]. The cardiotoxic effects of chemotherapeutic drugs commonly used in the ECT of tumors (bleomycin and cisplatin) could lead to changes in ECG [Tomirotti et al. 1984, Allen 1992, Villani et al. 1994, Tassinari et al. 1997, Bloom & Goldfarb 2005, Nuver et al. 2005, Yavas et al. 2008]. The cardiotoxicity can be indicated by appearance of or an increase in the incidence of premature atrial contractions, by appearance of supraventricular tachycardia, bradycardia or conduction abnormalities [Villani et al. 1994, Yavas et al. 2008]. Arrhythmias caused by chemotherapeutic drugs may occur during and shortly after drug administration by different mechanisms, such as direct effects of the drug on the heart, coronary artery

spasm and electrolyte imbalance [Yavas et al. 2008]. Furthermore, in cases when ECT is performed intra-operatively, some other drugs can also contribute to changes in functioning of the heart, such as anesthetics, analgesics and relaxants administered to the patient during the surgery.

Until now, no study on early and late effects of ECT on functioning of the heart has been performed. It is necessary to evaluate the relevance of this subject, especially due to increasing number of ongoing studies that are using ECT for treatment of deep seated tumors, and thus potentially close to the heart.

Several studies have shown that delivery of high voltage electrical pulses, like electroporation pulses, can lead also to major life-threatening irregularities in functioning of the heart (such as ventricular tachycardia with pressure drop, ventricular flutter and fibrillation) when they coincide with vulnerable period of the heart [Ayers et al. 1994, Lavee et al. 2007, Ball et al. 2010, Thomson 2010, Deodhar et al. 2011]. When electroporation pulses were delivered within thorax and near the heart outside the vulnerable period, no life-threatening changes in functioning of the heart were found [Deodhar et al. 2011]. However, in a study by Ayers et al., authors reported that the probability for ventricular fibrillation was decreased but not eliminated with the arrival of synchronized defibrillators for cardioversion [Ayers et al. 1994]. In theory, if time of electroporation pulse delivery with respect to vulnerable period of the heartbeat would be left out of consideration, a 33% chance exists that pulses would be delivered within the vulnerable period, because the duration of the vulnerable period is around one third of the duration of heartbeat [Reilly 1998]. This high percentage underlines the importance of synchronization of electroporation pulse delivery with ECG. To eliminate the possibility of electroporation pulse delivery during the vulnerable period of the heartbeat, the synchronization of electroporation pulses with the R wave of heartbeat should be used, as the R wave coincides with the safest period for pulse delivery (Figure 2) [Bertacchini et al. 2007, Bertacchini et al. 2010, Ball et al. 2010, Deodhar et al. 2011]. Cliniporator Vitae (Igea, Carpi, Italy), the new generation of the clinical device for ECT of deep seated tumors, provides basic synchronization capabilities [Bertacchini et al. 2010].

1.4. Effectiveness of electrochemotherapy

Although ECT is a local treatment of tumors, effectiveness of ECT is evaluated from clinician perspective as response per patient. For investigation of influence of different treatment conditions on effectiveness of ECT, response per tumor is, however, relevant. Tumor response can be determined following WHO or RECIST criteria [World Health Organization 1979, Therasse et al. 2000], or, alternatively, assessed by biopsy. Although WHO and RECIST criteria are different in some respects, these criteria are essentially equivalent for the evaluation of response of individual tumor lesions to treatment. Response of individual tumor is classified either as complete response (CR), partial response (PR), no change (NC) or progressive disease (PD). In RECIST criteria, classification of NC is replaced with stable disease (SD). In addition, the objective response (OR, including CR and PR) and the no response (NR, including NC and PD) classifications are frequently used. According to WHO and RECIST criteria, CR is defined as a disappearance of tumor, PR as a decrease of at least 50% in the products of the two largest perpendicular diameters of the tumor (corresponding to tumor area), and PD as an increase of more than 25% of lesion [World Health Organization 1979, Therasse et al. 2000]. In all other cases, a response is determined as NC. Tumor response has to be determined not earlier than four weeks after the treatment and confirmed not less than four weeks after the first evaluation. In clinical studies, complete and objective response rate (denoted as CR% and OR% respectively) are introduced for description of overall response of all treated tumors. Tumor response is regularly reported as response to a single-session ECT, taking into account that multiple ECT sessions are always possible and can only improve tumor response [Quaglino et al. 2008, Campana et al. 2009].

ECT has been reported as a highly effective antitumor therapy with CR% between 60 and 70% and OR% of about 80% [Sersa et al. 2008c, Marty et al. 2006, Sersa 2006, Landstrom et al. 2010, Kis et al. 2011, Matthiessen et al. 2011]. This effectiveness of ECT is relevant for single studies, among which large differences exist due to ECT performed under different treatment conditions. Several reviews have been reported on the effectiveness of ECT with aim to combine clinical outcomes from various individual studies and evaluate the overall effectiveness of ECT [Mir et al. 1998, Sersa 2006, Testori et al. 2008, Sadadcharam et al. 2008, Moller et al. 2009, Testori et al. 2010, Sersa et al. 2012, Testori et al. 2011]. However, there is a need to consolidate current experience on ECT treatment from the effectiveness point of view

and to establish the actual overall effectiveness of ECT derived from data reported in clinical studies to-date. Although clinical ECT is routinely used, some details about the effectiveness of ECT are still not fully understood and the procedure can be optimized.

The main prerequisites for effective ECT treatment are adequate extracellular concentration of the chemotherapeutic drug in the entire tumor at the time of electroporation pulse delivery, and coverage of tumor volume with sufficiently intense electric field able to permeabilize the cell membrane of tumor cells and therefore to enable the drug uptake [Domenge et al. 1996, Miklavcic et al. 1998, Miklavcic et al. 2000, Miklavcic et al. 2006]. Sufficiently high electric field in the tumor tissue can be assured by delivery of pulses of adequately high voltage and by appropriate positioning of the electrodes. In addition, some other independent and inter-related treatment conditions or parameters related to patient, tumor and treatment (such as age; gender; tumor histotype, size and location; drug type, dose and route of administration; electrode type; current, voltage and energy per volume delivered on tumor; protocol and timing of electroporation pulse delivery; median follow-up) probably contribute to variability in tumor response to ECT as well. In the literature, the histotype, size and location of the treated tumor, and the route of chemotherapeutic administration are the only mentioned parameters that seem to cause relatively large variability in effectiveness of ECT [Rodriguez-Cuevas et al. 2001, Rebersek et al. 2004, Marty et al. 2006, Larkin et al. 2007, Quaglino et al. 2008, Campana et al. 2009, Campana et al. 2012], but their role has not been sufficiently explored.

According to the literature, clear influence of different chemotherapeutic drugs (bleomycin and cisplatin) and the route of administration (intravenous and intratumoral) on effectiveness of ECT has not yet been determined. In early studies, higher effectiveness of ECT when using the intratumoral administration of bleomycin was reported than for the intravenous administration [Sersa 2006]. However, the results of ESOPE study showed comparable effectiveness of ECT for intravenous and intratumoral route of administration of bleomycin when given to tumors of volumes less than 0.5 cm^3, and that intravenous administration resulted in better antitumor effectiveness than intratumoral if tumors were bigger than 0.5 cm^3 [Marty et al. 2006]. For cisplatin, the studies on metastases of melanoma tumors have shown that the intravenous route is less effective than the intratumoral one [Sersa 2006]. Furthermore, the same effectiveness of ECT treatment was achieved when

comparing intratumoral administration of bleomycin and cisplatin. All these results, however, do not take into account the influence of other influential parameters. For example, it is not clear whether bleomycin or cisplatin work better on any tumor histotype or any tumor location.

The differences in effectiveness of ECT for different tumor histotypes were also observed, despite the general belief that effectiveness of ECT is equal regardless of tumor histotype. In summary, the highest effectiveness of ECT, with CR% of about 90% and OR% of about 100%, was shown in basal cell carcinoma patients regardless of drug type and administration [Fantini et al. 2008, Rodriguez-Cuevas et al. 2001, Sersa et al. 1998, Gargiulo et al. 2010, Heller et al. 1996, Heller et al. 1998, Landstrom et al. 2010, Mir et al. 1998]. On the other side, the lowest effectiveness, with CR% of about 50% and OR% of about 70%, was obtained for squamous cell carcinoma [Mir et al. 1998, Panje & Sadeghi 2000, Allegretti & Panje 2001, Burian et al. 2003, Bloom & Goldfarb 2005, Tijink et al. 2006, Gargiulo et al. 2010, Landstrom et al. 2010, Landstrom et al. 2011, Gargiulo et al. 2012]. Also according to the results of the ESOPE study, ECT was reported as equally effective regardless of the tumor histotype, although a trend towards higher effectiveness of ECT in non-melanoma versus melanoma tumors was detected (OR% of 90.4% versus 80.6%) [Marty et al. 2006].

The location of tumor was first found to have significant impact on effectiveness of ECT in ESOPE study, where ECT was more effective in tumor nodules located in the trunk in comparison to tumors located on limbs and in head and neck region [Marty et al. 2006]. On the contrary, in recent study by Campana et al. on malignant melanoma tumors, authors demonstrated that patients with tumors located on the limbs have more favorable prognosis than those with tumors located in the trunk [Campana et al. 2012].

Effectiveness of ECT varies also with respect to the size of treated tumors. In ESOPE study, where tumors only with diameters up to 3 cm were included, no statistical difference between the treatment response to ECT according to tumor size was found [Marty et al. 2006]. Since then, however, several clinical studies reported that ECT has been more effective in smaller than larger tumors, with limit value between smaller and larger tumors often set at 3 cm [Larkin et al. 2007, Quaglino et al. 2008, Campana et al. 2009, Campana et al. 2012].

One of the essential prerequisites for effective ECT is the coverage of entire tumor volume with electric field that is able to permeabilize the cell membranes and, therefore,

enables drug uptake. Different electrode types, voltages and protocols of electroporation pulses are currently being used. Besides known fact that sufficiently high electric field in the entire tumor must be assured in order to obtain effective ECT treatment, no other electrical parameter (such as minimal needed current or required energy applied on tumor volume) has been recognized that would enable to predict the treatment outcome. This issue was, however, addressed in ESOPE study, suggesting that for effective ECT with hexagonal needle electrodes the current has to exceed the value of 1.5 A, but no further examination regarding the optimization of electrical parameters, besides electrical field, has been recognized in the literature [Marty et al. 2006].

There exist many contradictions and ambiguities about the influence of these different treatment conditions and parameters on tumor response. A detailed investigation is thus needed that would identify and explain their influence on effectiveness of ECT. This knowledge could be used for the prognosis and further optimization of ECT protocols on cutaneous and subcutaneous tumors, as well as in development of ECT procedures for treating deep seated tumors, in order to maximize the probability for a successful outcome of ECT.

2. AIMS OF THE THESIS

Nowadays, electrochemotherapy (ECT) is included in clinical practice in more than 100 clinical centers and the use of ECT is rapidly growing. ECT is becoming not only the routine option for treatment of cutaneous and subcutaneous tumors [Marty et al. 2006, Moller et al. 2009, Testori et al. 2011, Sersa et al. 2012, Escoffre & Rols 2012], but also a viable option for treatment of various deep seated tumors [Soden et al. 2006, Miklavcic et al. 2010, Edhemovic et al. 2011, Pavliha et al. 2012]. However, some details about the safety and effectiveness of ECT are still not sufficiently clarified and there is still room for further optimization of the procedure.

The aims of this doctoral thesis cover three important issues concerning clinical ECT:

- evaluation of safety of clinical ECT, in context of the potential influence on functioning of the heart;
- development and evaluation of the algorithm for synchronization of electroporation pulses with electrocardiogram (ECG); and
- evaluation of effectiveness of clinical ECT and its dependence on treatment conditions.

2.1. EVALUATION OF SAFETY OF CLINICAL ELECTROCHEMOTHERAPY

The aims of this topic of our study are:

- to examine theoretical possibility of influence of ECT of cutaneous and subcutaneous tumors on functioning of the heart;
- to address the relevance of synchronization of electroporation pulse delivery with ECG;
- to evaluate early (occurring during and immediately after ECT treatment) effects of clinical ECT of cutaneous and subcutaneous tumors on functioning of the heart;
- to evaluate early and late (occurring within 24 hours after ECT treatment) effects of clinical intra-abdominal ECT of tumors in liver on functioning of the heart;
- to assess if changes (if any) in different evaluated parameters derived from ECG signal are correlated with electrical parameters (current, energy) or treatment conditions (electrode configuration, protocol of electroporation pulse delivery) used during ECT.

2.2. Development and evaluation of the algorithm for synchronization of electroporation pulses with electrocardiogram

The aims of this topic of our study are:

- to develop algorithm for effective and reliable synchronization of electroporation pulse delivery with ECG that will enable real-time implementation;
- to evaluate the algorithm for synchronization of electroporation pulse delivery with ECG on ECG signals from the standard database and on ECG signals recorded during clinical ECT procedure;
- to perform the simulation of synchronized electroporation pulse delivery on ECG signals recorded during clinical ECT and to compare the results of our simulation with the performance of synchronization protocol currently implemented in clinical device for ECT.

2.3. Evaluation of effectiveness of clinical electrochemotherapy and its dependence on treatment conditions

The aims of this topic of our study are:

- to determine an overall effectiveness of single-session ECT of cutaneous and subcutaneous tumors regardless of different treatment conditions;
- to determine effectiveness of ECT in comparison to effectiveness of chemotherapeutic drug alone;
- to evaluate differences in effectiveness of ECT with respect to drug type and route of administration;
- to evaluate differences in effectiveness of ECT with respect to histotype of tumors;
- to evaluate differences in effectiveness of ECT with respect to tumor size;
- to suggest further steps in refinement of clinical ECT that could maximize the probability of successful outcome of ECT of cutaneous and subcutaneous tumors but also to the successful development of ECT for deep seated tumors.

3. MATERIALS AND METHODS

In this chapter, we briefly outline the materials and methods on the three important issues concerning clinical electrochemotherapy (ECT) according to the aims of our study. The detailed description of materials and methods used in our study is presented in the form of papers in the Appendix of the thesis.

3.1. EVALUATION OF SAFETY OF CLINICAL ELECTROCHEMOTHERAPY

3.1.1. NUMERICAL MODELING

Different numerical models of tumors in muscle tissue (representing heart muscle) were built with geometry and electrode configurations mimicking those used in clinical ECT. Three different types of electrodes were modeled: plate, needle row array and needle hexagonal array. Numerical calculations of electric field and current distribution for tissue models were performed at different distances between the plate electrodes and at different depths of insertion of needle electrodes. The critical depth for reversible and irreversible electroporation of the muscle (i.e. heart) and critical depth for current of 100 mA, i.e. threshold for ventricular fibrillation, were estimated by means of finite element method using COMSOL Multiphysics 3.3 software package (COMSOL AB, Sweden). Critical depth, defined as depth below which the total electric current flowing is equal to the threshold value for fibrillation, was calculated for model with different geometries and voltages applied. This critical depth served as an estimate for depth, below which we do not expect any critical side effect when performing ECT treatment of cutaneous and subcutaneous tumors with such types of electrodes. For details, see **Paper I**.

3.1.2. ECG SIGNALS AND ECT TREATMENT DATA

Changes reflecting on ECG due to ECT procedure were examined using ECG signals recorded during clinical procedures at the Institute of Oncology Ljubljana. Two different systems were used for ECG recording: Biopac system (Biopac Systems, Inc., USA) for ECG recording during ECT treatment and Holter system (SpiderView, ELA Medical, France) for long-term ECG monitoring before and after ECT treatment (Figure 3). The relatively short

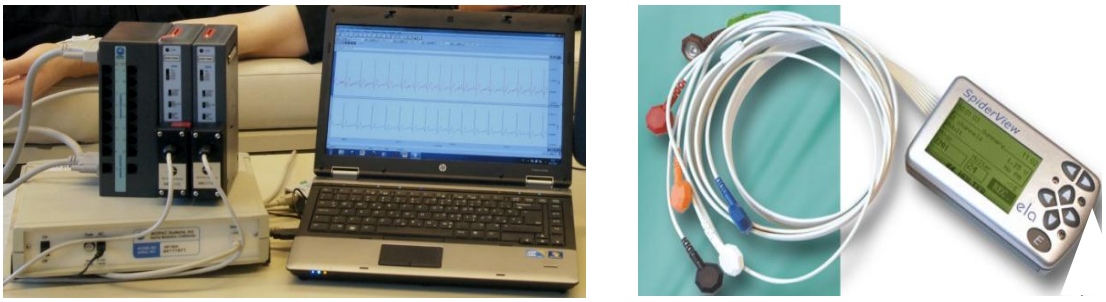

Figure 3: The systems for ECG signal recording: Biopac system (left) and Holter system (right).

(approximately 2-hour-long) ECG recordings during ECT treatment of cutaneous and subcutaneous or deep seated tumors enabled extraction of early (occurring during and immediately after ECT treatment) effects of ECT on functioning of the heart (**Paper I**, **Paper II**, **Paper IV**), whereas longer (preferably 24-hour-long) Holter ECG recordings before and after intra-abdominal ECT of deep seated tumors were used for the evaluation of late (occurring within 24 hours after ECT treatment) effects on functioning of the heart (**Paper III**). Altogether, 16 ECG signals were recorded during ECT of cutaneous and subcutaneous tumors, 18 ECG signals during intra-abdominal ECT of tumors (mainly colorectal carcinoma metastases) in liver performed under general anesthesia, and 11 ECG signals before and after intra-abdominal ECT of tumors in liver. For details, see **Paper I**, **Paper II**, **Paper III** and **Paper IV**.

For every ECT treatment performed on each tumor and patient, ECT treatment data including type of electrode used, values of set treatment parameters (voltages, frequency, duration and number of pulses) and time course of voltage and current during electroporation pulse delivery were recorded on the Cliniporator device. In addition, different patient and tumor characteristics and treatment conditions (such as age; gender; histotype, size and location of tumors; drug type, dose and administration route; timing of pulse delivery) were also collected.

3.1.3. Analysis of ECG signals and ECT treatment data

The algorithm for detailed post-analysis of recorded ECG signals was developed in ANSI C programming language and Matlab (**Paper I**, **Paper V**). The algorithm consists of a learning phase, a QRS detector and a routine for classification of heartbeats. The algorithm adapts architectural parameters to characteristics of the analyzed ECG signal and extracts important individual heartbeat characteristics (Figure 4) and overall characteristics of ECG

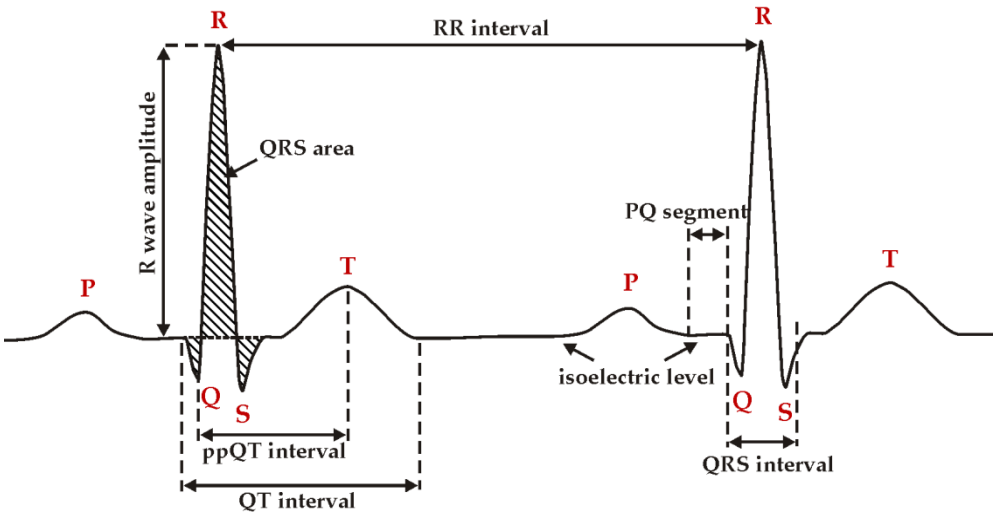

Figure 4: Characteristics of a typical heartbeat.

signal with different approaches (e.g. averages of different heartbeat intervals, such as RR and corrected QT interval, over different time intervals (**Paper I**, **Paper IV**); heart rate variability analysis in time and frequency domain and nonlinear analysis (**Paper II**, **Paper III**); prediction interval for values of various parameters derived from ECG signal (**Paper II**)). Heartbeat and overall ECG characteristics are used for the quantitative evaluation of changes during and after ECT. Special software with graphical user interface (Figure 5) was additionally developed in Matlab for easier screening and checking for correctness of the located feature points (Q, R and T peak locations) and classification of heartbeats (**Paper III**).

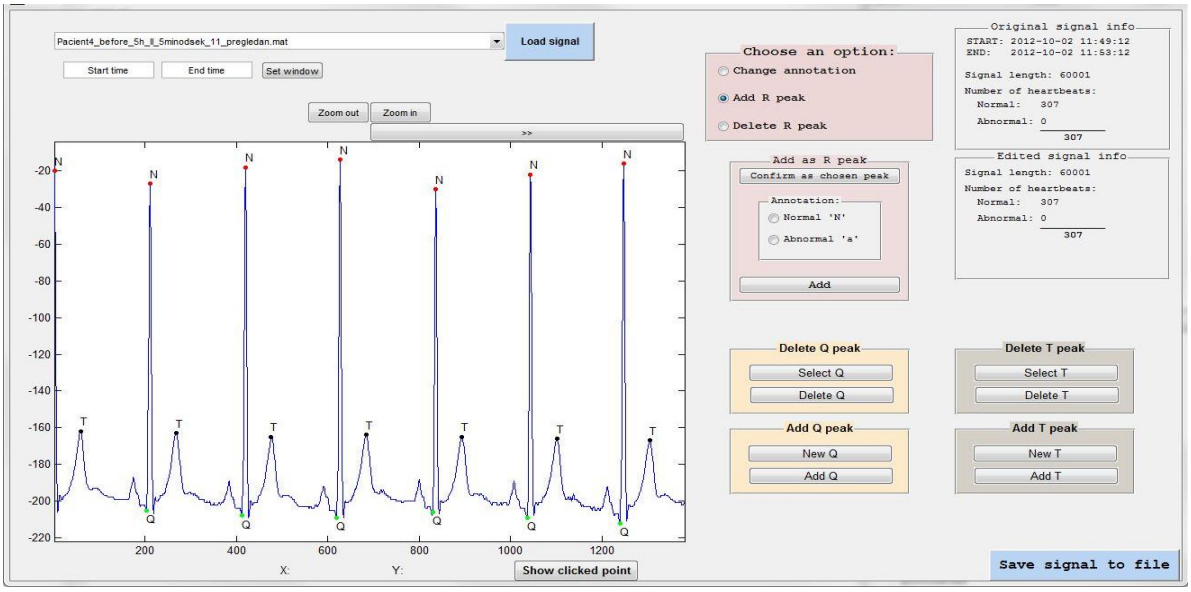

Figure 5: A graphical user interface of a program developed in Matlab for manual editing of Q, R and T peak locations.

Analysis of ECT treatment data recorded on Cliniporator device, as well as other data about the ECT treatment, was performed using software developed in Matlab. This software enabled to establish the average values of voltage and current, the calculations of energy applied on each tumor (i.e. integral of product of current and voltage delivered during electroporation pulse delivery on tumor) and total energy applied on individual patient (i.e. the sum of values of energy applied on individual tumors of a patient), and correlations between different data sets. In addition to this specially designed software, commercially available statistical packages were also used (Statistical Toolbox in Matlab, SigmaPlot, Excel), for execution of various ordinary statistical tests (e.g. t-test, ANOVA, Chi-square, correlation and linear regression).

For details on topic of this subchapter, see **Paper I**, **Paper II**, **Paper III** and **Paper IV**.

3.2. Development and evaluation of the algorithm for synchronization of electroporation pulses with electrocardiogram

3.2.1. Development of the algorithm

In the process of developing the algorithm, we used two 2-hour-long sections of ECG signals from the Long-Term ST (LTST) database [Jager et al. 2003]. The algorithm is based on time-domain analysis of a single ECG lead sampled at 250 Hz. It searches for the initial portion of the QRS complex, i.e. the ascendant QR junction slope and R wave peak, as early as possible before the vulnerable period, thus leaving enough time within QRS complex for electroporation pulse delivery. The algorithm consists of two major components (the QRS detection phase and the decision-making phase), which are preceded by the learning phase (Figure 6). The algorithm thus enables adjustment of its architectural parameters to the characteristics of an analyzed ECG signal, early detection of QRS complexes (based on the first and the second signal derivative), classification of heartbeats as normal or abnormal (based on amplitude of R wave, duration of RR interval and deviations of extracted characteristics of individual heartbeats from average values), and decision-making about the synchronized delivery of electroporation pulses (only outside of the vulnerable period and in absence of heart arrhythmias). The algorithm was written in ANSI C programming language and designed in a way that enables real-time implementation. For details, see **Paper V**.

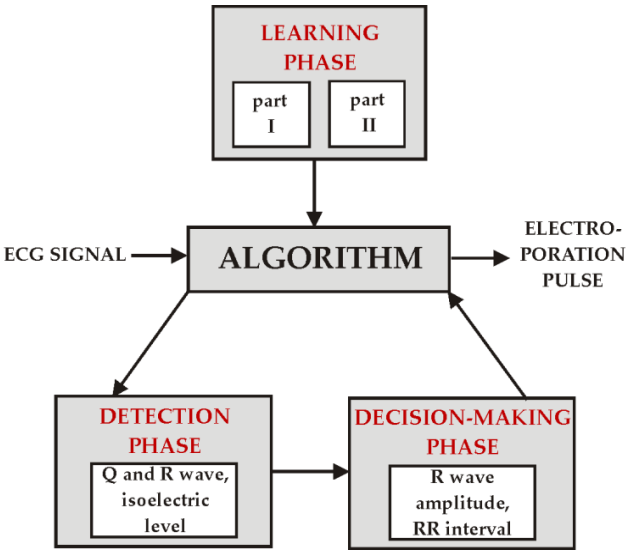

Figure 6: The structure of the algorithm for synchronization of electroporation pulses with ECG.

3.2.2. Evaluation of the algorithm

Initially, the performance of the developed algorithm for synchronization of electroporation pulses with ECG was evaluated on 42 24-hour-long ECG signals from the LTST database [Jager et al. 2003]. The sampling frequency of the signals was 250 Hz. The LTST database contains ambulatory Holter records reflecting real-world clinical environment with all heartbeats classified and annotated by experts. Therefore, all usual daily activities of the patient are reflected in these ECG signals, which also contain abnormal heartbeats of various pathological backgrounds. Annotated heartbeats allowed reliable evaluation of the algorithm with different standard performance metrics (sensitivity, positive predictivity and detection error rate for QRS detection and for electroporation pulse delivery). For details, see **Paper V**.

Afterwards, the performance of the algorithm was tested on 16 ECG signals (durations between 10 and 30 min) recorded during clinical ECT of cutaneous and subcutaneous tumors at Institute of Oncology Ljubljana. ECT was performed in local anesthesia when patient was resting comfortably. Performance metrics (sensitivity, positive predictivity and detection error rate for QRS detection and for electroporation pulse delivery) were again calculated in order to evaluate functioning of the algorithm under real clinical conditions. For details, see **Paper I**.

3.2.3. Performance of our algorithm in comparison with synchronization protocol currently used in clinical electrochemotherapy

The performance of our synchronization algorithm was assessed by simulation of synchronized pulse delivery on three approximately 2-hour-long ECG signals recorded during intra-abdominal ECT of tumors in liver. The simulated starting times of electroporation pulse delivery were the same as actually appeared in real ECT application. Together with ECG signals, R trigger signals obtained from ECG triggering device AccuSync 42 (AccuSync Medical Research Corp., Milford, CT, USA) were also stored for evaluation of performance of algorithm for synchronization currently implemented in Cliniporator Vitae. The comparison of both results (from our and currently implemented synchronization algorithm) was carried out, which enabled to address potential weaknesses and to suggest changes for improvement of the existing protocol for clinical ECT in the future. For details, see **Paper II**.

3.3. Evaluation of effectiveness of clinical electrochemotherapy and its dependence on treatment conditions

3.3.1. Search for and extraction of eligible data

For the purpose of evaluation of clinical effectiveness of ECT, the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed [Liberati et al. 2009, Moher et al. 2009]. The identification of studies eligible for our study was executed with systematic search of 16 biomedical bibliographic databases and reference lists of reviews.

Clinical studies were included in our study if the following criteria were met: (1) inclusion of human patients requiring therapy of cutaneous or subcutaneous tumors of any histotype; (2) treatment of tumors performed with single-session ECT using either bleomycin or cisplatin administered intratumorally or intravenously; (3) studies including information about number of patients and tumors, tumor response, the chemotherapeutic drug used, the route of drug administration and type of tumor; and (4) the response of tumors must had been evaluated at least 4 weeks after the treatment. Clinical studies were eligible for meta-

analysis if they met any of the further three criteria in addition to those listed above: (5) use of control tumors that where either tumors treated with chemotherapeutic drug only or electroporation pulses only, or tumors that received no treatment at all; and/or (6) treatment of at least two different tumor histotypes.; and/or (7) treatment of tumors with diameters smaller and bigger than 3 cm.

The data extracted from the studies included study design type, number and gender of patients, number of tumors, histotype of tumors, size of tumors, chemotherapeutic drug, route of drug administration, outcome data (tumor response) for treated and control tumors (if controls were used), criteria for tumor response evaluation, duration of follow-up and assessment of risk of bias. Data extraction was carried out using independent screening process of at least two persons.

The search strategy within bibliographic databases using search terms 'electrochemotherapy' and 'clinical' identified more than 1100 articles. After the exclusion of irrelevant studies, 44 studies were identified as appropriate for further systematic review and data analysis. However, only smaller subsets of this group of studies were eligible for meta-analyses: 13 studies for comparison of antitumor effectiveness between ECT and chemotherapy alone, 8 studies for comparison of tumor response to ECT between melanoma and non-melanoma, 6 studies for comparison of tumor response to ECT between carcinoma and melanoma tumors, and 9 studies for comparison of tumor response between smaller and larger tumors. For the latest comparison between different tumor sizes, two different databases of ECT of cutaneous and subcutaneous tumors were also identified as appropriate for our analysis. The first database with a full access to all patient, tumor and ECT treatment data was collected at the Institute of Oncology Ljubljana (denoted as IO data). The second database included the raw data about tumor size and response, and was collected at Veneto Region Oncology Research Institute from Padova in Italy (denoted as CP data). For details, see **Paper VI** and **Paper VII**.

3.3.2. Analysis of data

The analysis of extracted data from all eligible studies was first carried out by merging the data about CR and OR from individual tumors and calculating the overall CR% and OR%, which enabled determination of overall effectiveness of ECT, regardless of different

treatment conditions and parameters used. Similar procedure was used also for calculation of effectiveness under different specific treatment conditions; for example, calculation of effectiveness when data of specific histotype of tumor were merged, or when data regarding specific chemotherapeutic drug and route of administration were merged. Chi-square test was applied to determine if the difference of CR and OR proportions between different groups (such as different histotypes of tumors) was statistically significant.

Meta-analysis was performed on smaller subsets of studies that included comparable data within the study (see previous section). Meta-analysis is generally the preferred method for pooling the results of independent studies. Risk difference, defined as probability of response (either CR or OR) in one group minus the probability of the same response in the other group, was used as the measure of the effect because of dichotomous nature of tumors' response data. The summary effect of meta-analysis was combined using the so-called random-effects model. Consequently, larger studies (with many tumors) do not dominate the overall effect as much and smaller studies (with few tumors) are not trivialized as would be the case with the so-called fixed-effects model. The software for meta-analysis calculations and figures with the so-called forest plots (Figure 7) was developed in Matlab.

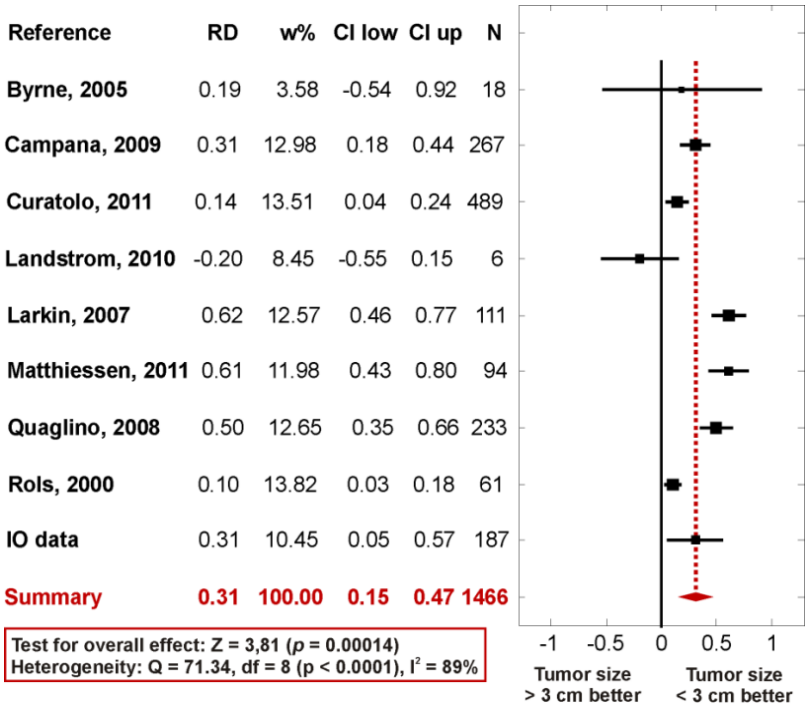

Figure 7: Example of meta-analysis results presented using forest plot. RD = individual and summary risk difference for studies included in meta-analysis. w% = weight of study in comparison to all studies. CI low and CI up = the lower and upper confidence interval of RD, respectively. N = the number of tumors per each study and total number of tumors included in meta-analysis.

On the raw data accessible from IO and CP databases, the ordinary statistical tests were employed (e.g. t-test, ANOVA, Chi-square, correlation and linear regression) using different statistical packages (Statistical Toolbox in Matlab, SigmaPlot, Excel), in order to determine treatment conditions and parameters for effective clinical ECT. The methods of knowledge discovery were applied to these data using different approaches implemented in Matlab Statistical Toolbox (e.g. cluster analysis, decision trees, different types of statistic tests, principal component analysis, factor analysis) to identify patterns within these sets of clinical data in an attempt to connect treatment conditions and parameters to the treatment outcome.

For details on this topic, see **Paper VI** and **Paper VII**.

4. RESULTS AND DISCUSSION

In this chapter, we outline the results and discuss three outstanding issues concerning clinical electrochemotherapy (ECT) according to the aims of our study. The detailed description of the results and thorough discussions are presented in the form of papers in the Appendix of the thesis and only brief summary is given here below.

4.1. EVALUATION OF SAFETY OF CLINICAL ELECTROCHEMOTHERAPY

The results concerning safety of clinical ECT of cutaneous and subcutaneous tumors are presented in **Paper I**.

In **Paper I**, numerical calculations of electric field and current distribution for mathematical models, presenting the geometry, electrode configurations and conditions used during ECT of cutaneous and subcutaneous tumors, indicated that it is highly unlikely that electroporation pulses applied distantly from the heart, such as on upper or lower limbs, could have any influence on functioning of the heart. However, this is not necessary the case for electroporation pulses applied on the chest. Electroporation pulses delivered on the chest using electrodes located above sternum or above a rib have a very low probability of affecting the heart, due to low conductivity of bones and larger dimension of sternum and ribs in comparison to the distance between the electrodes. However, if the electrodes are not located directly above the sternum or a rib, the critical depth (i.e. depth below which we do not expect any critical heart-related effect) will be larger due to higher conductivity of the underlying tissues. In this case, according to the results of modeling, there is a larger theoretical possibility to affect functioning of the heart in case of deep insertion of either needle row array or hexagonal array electrodes (two types of electrodes used for clinical ECT), taking into account that heart lies at least 3 cm beneath the skin surface. Namely, the critical depths are 3.8 cm and 4.1 cm for needle row array and hexagonal array electrodes, respectively, at insertion depth of 10 mm. This aspect should be considered in the future applications of clinical ECT. The use of synchronization of electroporation pulse delivery with ECG is therefore also advised.

In **Paper I**, the analysis of electrocardiogram (ECG) signals recorded on patients subjected to clinical ECT of cutaneous and subcutaneous tumors under local anesthesia revealed no pathological morphological changes in ECG, even though the electroporation pulses were not synchronized with ECG. No early effects expressed as heart arrhythmias due to electroporation pulse delivery were induced. Moreover, the incidence of premature heartbeats was not increased by electroporation pulses. However, the program-based analysis of heartbeats characteristics (RR interval, QRS interval, QTc interval, R wave and QRS area) detected the early effect manifested as a significant decrease in RR and QRS interval duration (reflecting an increase in heart rate) after each application of electroporation pulses. We suggest, however, that the observed transient decrease in RR interval can be largely, if not completely, attributed to anxiety and stress of the patient undergoing ECT.

On the other side, as stated in **Paper I**, some open safety issues regarding ECT still need to be considered, for example: electroporation pulses delivered using plate or needle row array electrodes that are not synchronized with ECG can be delivered within the vulnerable period; delivery of pulses using needle hexagonal array electrodes inevitably coincides within the vulnerable period due to much longer pulse delivery protocol (approximately 200 ms); the threshold levels of the heart for elderly patients are significantly lower than in younger population; possible use of ECT on patients with clinically significant heart disease; new applications with longer durations or larger number of pulses of increased pulse repetition frequency and/or higher amplitudes of pulses and/or in the treatment of tumors on the chest close to the heart.

Considering the results in **Paper I**, although no serious consequences on functioning of the heart (such as induction of various arrhythmias or, in the worst case, ventricular fibrillation) have been recorded due to ECT procedure on cutaneous and subcutaneous tumors until now, it is nevertheless advisable to avoid delivery of electroporation pulses during the vulnerable period and in case of heart arrhythmias. In order to maximize safety of the patient, synchronization of electroporation pulse delivery with the refractory period of the cardiac cycle should be incorporated in medical equipment for ECT.

The results on safety of intra-abdominal ECT of tumors in liver are presented in **Paper II**, **Paper III** and **Paper IV**.

In **Paper II** and **Paper IV**, the results of analysis of ECG signals recorded during intra-abdominal ECT of tumors in liver demonstrate early effect of administration of bleomycin, expressed as occurrence of premature atrial contractions immediately during intravenous administration. In addition, during synchronized electroporation pulse delivery, we found a significant transient decrease in duration of the corrected QT interval. Electroporation pulse delivery also increased short-term heart rate variability parameters: Poincaré plot descriptor SD1 and time-domain parameter SDSD. This early effect appeared most likely due to effect of electric stimulation of surrounding muscles and nerves caused by electroporation pulses, which consequently provoke cardiovascular responses, commonly manifested as transient increase in heart rate. In relation to these results, we suggest that it is possible to affect functioning of the heart by intra-abdominal ECT of deep seated tumors, despite synchronized electroporation pulse delivery, but most probably these changes can only be transient. No adverse effects due to intra-abdominal ECT were found, which may be due to synchronized electroporation pulse delivery with the ECG, but the probability for complications could increase when using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or in the treatment of tumors in the immediate vicinity of the heart. For this reason, when deep seated tumors are treated, the synchronization of electroporation pulses with ECG should be mandatory, in order to maximize the safety of the patients.

The results in **Paper III** based on the analysis of ECG signals recorded before and after intra-abdominal ECT of tumors in liver indicate that the entire procedure of intra-abdominal ECT has some late effects on heart rate and long-term HRV. However, no major late (occurring within 24 hours after ECT treatment) effects (i.e. ventricular tachycardia or fibrillation) on functioning of the heart of patients or post-operative heart arrhythmias due to intra-abdominal ECT were identified. Intra-abdominal ECT has also no late effects expressed as change of ST segment. We identified statistically significant decrease in proportion of abnormal beats. Based on HRV analysis, statistically significant decrease in median values of mean NN interval, SD2, LF and nLF, and increase in nHF were observed after intra-abdominal ECT. Currently, it is not clear whether these detected changes in HRV measures appeared due to ECT procedure alone or due to effects of post-operative pain and drugs administered in post-operative care. Further study to clarify this question is needed.

In **Paper II** and **Paper III**, we estimated correlation between changes in different evaluated parameters derived from ECG signal, and electrical parameters (current, energy) or treatment conditions (electrode configuration, protocol of electroporation pulse delivery) used during ECT. In **Paper II** on topic of early effects of intra-abdominal ECT, we found statistically significant correlation between changes in corrected QT interval and the average amplitude of electrical current and energy applied during electroporation pulse delivery. The linear regression between change in RR interval and current was also found statistically significant. In **Paper III**, results showed statistically significant negative correlation between changes in SD2 and LF component, and the number of delivered electroporation pulses on a patient. Comparison of different protocols of electroporation pulse delivery (bipolar and unipolar delivery of electroporation pulses using needle electrodes with custom geometry, and bipolar delivery of electroporation pulses using needle electrodes with hexagonal geometry) indicated no statistically significant differences.

4.2. Development and evaluation of the algorithm for synchronization of electroporation pulses with electrocardiogram

The algorithm for synchronization of electroporation pulses with electrocardiogram is described in **Paper I, Paper II** and **Paper V**.

In **Paper V**, we present the developed algorithm which can be used for safer use of ECT, as well as non-thermal irreversible electroporation tissue ablation treatment and other treatments that require delivery of high-voltage pulses on patient. The evaluation of the algorithm's performance was performed in two ways: on 42 24-hour-long records of the Long-Term ST (LTST) database and on 16 ECG signals recorded during clinical ECT of cutaneous and subcutaneous tumors. The evaluation of the algorithm on ECG signals from LTST database illustrates performance of the algorithm in real-world conditions, because all usual daily activities of the patient are reflected in these ECG signals that also contain abnormal heartbeats of various pathological backgrounds. ECG signals recorded during clinical ECT are not expected to involve lots of such ECG anomalies.

Based on evaluation of the algorithm on LTST database described in **Paper V**, the algorithm correctly detected 99.4% (sensitivity) of all QRS complexes, on average. The

detection error rate was 0.6%, and average positive predictivity for QRS detection was 100.0%. The algorithm proved to be an effective tool for QRS detection, which is a prerequisite condition for accurate and reliable electroporation pulse delivery. The algorithm proved to deliver electroporation pulses only outside the vulnerable period and to prevent pulses from being delivered in case of appearance of some heart arrhythmias, such as atrial and ventricular premature beats. The results of the evaluation, a sensitivity of 91.8%, a positive predictivity of 100.0% and a delivery error rate of 8.3% for electroporation pulse delivery (medians), suggest that the algorithm is accurate and appropriate for application in ECT of tumors regardless of tumor location. The algorithm is designed for robust operation even in cases of numerous heart arrhythmias. The performance of the algorithm is significantly degraded only in presence of disturbances due to body movements with morphologies similar to morphology of QRS complex. The algorithm delivers electroporation pulse(s) immediately after the QRS detection but still within the QRS complex. The estimated time reserve for safe electroporation pulse delivery after the QRS detection and before the onset of vulnerable period for ventricles is approximately 60 ms and is long enough for safe electroporation pulse delivery, even if we want to avoid the vulnerable period of atria as well.

In **Paper I**, the performance of the algorithm was tested on 16 ECG signals recorded during clinical ECT of cutaneous and subcutaneous tumors. On average, the algorithm correctly detected 99.2% of all QRS complexes and would correctly deliver electroporation pulses in 94.6% of normal QRS complexes. The average positive predictivity for electroporation pulses was 100.0%, and thus ideal. This performance measure was significantly better for ECG signals recorded during ECT than for ECG signals from LTST database, most likely due to fewer ECG anomalies encountered in ECG signals recorded during clinical application of ECT than in ECG signals from LTST database.

The simulation of synchronized pulse delivery on ECG signals recorded during intra-abdominal ECT of tumors in liver and comparison of the results with the performance of synchronization protocol currently implemented in clinical device for ECT is described in **Paper II.** Altogether 240 electroporation pulses were delivered (in reality and in the simulation) on three patients included in analysis. The results show that currently implemented algorithm for synchronization of electroporation pulses with ECG in

Cliniporator Vitae delivered 3 electroporation pulses at wrong positions (one on a P wave, one just before a P wave, and one right after a T wave) and would deliver pulses in case of every premature atrial and ventricular contraction. Our own algorithm, however, did not deliver electroporation pulse on wrong positions and would not deliver electroporation pulses on premature beats. Even though our algorithm is more reliable, it would not introduce any significant or clinically relevant increase in duration to the entire electroporation pulse delivery procedure. Finally, we summarize that currently implemented synchronization protocol in Cliniporator Vitae does not guaranty the delivery of electroporation pulses exclusively on the R waves (or outside the vulnerable period of the heart). We suggest that the validity of current R trigger signal from AccuSync device should be determined based on an on-line analysis of current RR interval with respect to a regularly updated running average of eight previous RR intervals. For details, see **Paper II**.

4.3. Evaluation of effectiveness of clinical electrochemotherapy and its dependence on treatment conditions

The results of evaluation of effectiveness of clinical ECT are presented in **Paper IV**, **Paper VI** and **Paper VII**.

For evaluation of effectiveness of single-session ECT of cutaneous and subcutaneous tumors in **Paper VI**, 44 studies involving 1894 tumors were included. The overall CR% and OR% of 59.4% and 84.1%, respectively, were calculated for ECT, and of 8.0% and 19.9%, respectively, for treatment with bleomycin or cisplatin alone. Similarly, the results of meta-analysis show that electroporation pulse delivery significantly potentiates the effectiveness of chemotherapeutic drug alone by more than 50%. In **Paper IV**, the feasibility and effectiveness of intra-abdominal ECT of tumor in liver was confirmed. The tumor responded to single-session ECT treatment with CR.

The effectiveness of ECT of cutaneous and subcutaneous tumors was found significantly higher for intratumoral (CR% and OR% of 72.7% and 85.8%, respectively) than for intravenous administration of bleomycin (CR% and OR% of 54.9% and 80.7%, respectively). Bleomycin and cisplatin administered intratumorally resulted in equal effectiveness of ECT.

According to our results described in **Paper VI**, the often repeated statement about equal clinical effectiveness of ECT regardless of tumor histotype appears to be unjustified. Namely, non-melanoma tumors (all non-melanoma types lumped together) responded significantly better to ECT (CR% and OR% of 67.0% and 86.4%, respectively) than melanoma tumors (CR% and OR% of 56.8% and 80.6%, respectively). ECT was more effective in sarcoma than in melanoma or carcinoma tumors. Among carcinoma tumors (i.e. squamous cell carcinoma, basal cell carcinoma, adenocarcinoma), basal cell carcinoma tumors yield significantly better response than melanoma tumors. Among all histotypes of tumors, basal cell carcinomas has the highest and squamous cell carcinomas the lowest overall CR% and OR%, which, however, might be attributed to their different sizes; namely, the squamous cell carcinoma tumors were usually significantly larger than basal cell carcinoma and would, therefore, require repeated ECT treatments. A statistical comparison of response between different tumor histotypes (melanoma, carcinoma and sarcoma), separately for each chemotherapeutic drug and route of administration, indicate that bleomycin and cisplatin work with similar effectiveness regardless of tumor histotype. Similarly, a statistical comparison of response between different chemotherapeutic drugs and routes of drug administration, separately for each tumor type, show that none of tumors respond differently to the same chemotherapeutic drug and route of administration.

Significantly lower effectiveness of ECT of cutaneous and subcutaneous tumors with maximal diameter equal to or larger than 3 cm (CR% of 33.3%, OR% of 68.2%) in comparison to smaller tumors (CR% of 59.5%, OR% of 85.7%) was found in **Paper VII**. The analysis of raw size and response data of cutaneous and subcutaneous tumors showed statistically significant decrease in effectiveness of single-session ECT progressively with increasing tumor diameter. Tumor size started to play a significant role in the final treatment outcome for tumors as small as about 2 cm in diameter. It remains to be determined if observed lower effectiveness on larger tumors is due to the size alone or due to other treatment conditions and parameters. There are several possible explanations for this observed effect: inadequate concentration of chemotherapeutic drug reached in the target tumor due to improper timing of electroporation pulse delivery or due to large temporal and spatial heterogeneity in blood flow of tumors, or insufficient coverage of the entire tumor volume with sufficiently high electric field. We suggest that standard operating procedures for ECT should be reexamined

and refined for treatment of larger tumors. We propose that future clinical trials should include accurate ECT treatment planning or application of fixed-geometry electrodes with their accurate repositioning in order to overlap the treated volumes and/or multiple ECT cycles, besides a prolonged observation for tumor response evaluation. Bleomycin could be administered intravenously and intratumorally to achieve sufficient extracellular concentration in the portion of the tumor.

The results demonstrated in **Paper VI** and **Paper VII** show that the differences in effectiveness of ECT depend significantly on treatment conditions. The results of our systematic review shed new light on effectiveness of ECT and can be used for prediction of tumor response to ECT with respect to various treatment conditions and should be taken into account in further refinement of ECT protocols on cutaneous and subcutaneous tumors as well as in development of ECT procedures for treating deep seated tumors.

5. CONCLUSIONS AND FUTURE PROSPECTS

In this chapter, we outline the conclusions on three outstanding issues concerning safety and effectiveness of clinical electrochemotherapy (ECT) according to the aims of our study. Details of these issues are described in the form of papers in the Appendix of the thesis.

5.1. EVALUATION OF SAFETY OF CLINICAL ELECTROCHEMOTHERAPY

5.1.1. NUMERICAL MODELING

- The results of numerical modeling of electric field and current distribution show that a theoretical possibility for an effect of electric pulses used in ECT on functioning of the heart exists in case of deep insertion of either row array or hexagonal array needle electrodes, but not for plate electrodes [**Paper I**]. The critical depth of possible harmful effects of electroporation pulses on functioning of the heart is estimated to be around 4 cm for needle electrodes at insertion depth of 10 mm. This aspect should be considered in the future applications of clinical ECT. The use of synchronization of electroporation pulse delivery with electrocardiogram (ECG) is also recommended.

5.1.2. CLINICAL ELECTROCHEMOTHERAPY OF CUTANEOUS AND SUBCUTANEOUS TUMORS

- Clinical ECT of cutaneous and subcutaneous tumors does not induce pathological morphological changes or heart arrhythmias due to unsynchronized electroporation pulse delivery [**Paper I**].
- Clinical ECT of cutaneous and subcutaneous tumors induces significant transient decrease in RR and QRS interval duration, i.e. increase in heart rate, after each unsynchronized application of electroporation pulses, which can be largely, if not completely, attributed to anxiety and stress of the patient undergoing ECT [**Paper I**].
- In conclusion, functioning of the heart by ECT performed on cutaneous and subcutaneous tumors can be affected, particularly due to unsynchronized electroporation pulse delivery, but these changes will probably be transient. However, the probability for

harmful effects of ECT on functioning of the heart could increase when using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or in the treatment of tumors on the chest close to the heart. For this reason, when cutaneous and subcutaneous tumors are treated, the synchronization of electroporation pulses with ECG is nevertheless advisable, in order to maximize the safety of the patients [**Paper I**].

5.1.3. Intra-abdominal electrochemotherapy of tumors in liver

- No major early or late effects (i.e. ventricular tachycardia or fibrillation) due to intra-abdominal ECT were found, which may be thanks to synchronization of electroporation pulse delivery with ECG [**Paper II**, **Paper III**, **Paper IV**].
- Early effect of administration of bleomycin, expressed as transient occurrence of premature atrial contractions immediately during intravenous administration, was demonstrated from ECG signals recorded during intra-abdominal ECT of tumors in liver [**Paper II**].
- Early effect of synchronized electroporation pulse delivery, manifested as transient decrease in the corrected QT interval and increased short-term heart rate variability (HRV), was detected from ECG signals recorded during intra-abdominal ECT of tumors in liver [**Paper II**, **Paper IV**].
- Late effects manifested as increased heart rate (i.e. decreased NN interval) and nHF, and decreased long-term HRV parameters SD2, LF and nLF were found from ECG signals recorded before and after intra-abdominal ECT of tumors in liver [**Paper III**]. Currently, however, it is still not clear whether these detected changes in HRV measures appeared due to ECT procedure alone or due to effects of post-operative pain and drugs administered in post-operative care.
- We conclude that it is possible to affect functioning of the heart by intra-abdominal ECT of tumors in liver, despite synchronized electroporation pulse delivery used, but most probably these changes would be transient [**Paper II**, **Paper III**, **Paper IV**]. However, the probability for harmful effects of intra-abdominal ECT could increase when using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or in the treatment of tumors in the immediate vicinity of the heart. For this reason,

when deep seated tumors are treated, the synchronization of electroporation pulses with ECG should be mandatory, in order to maximize the safety of patients.

5.1.4. Future prospects on safety of clinical electrochemotherapy

In our future work, we need to expand search of possible effects of intra-abdominal ECT on some other indices, which have not yet been included in the evaluation, in order to clarify if observed late effects of surgery procedure including intra-abdominal ECT of tumors in liver can be attributed also to ECT procedure alone. For example, changes in T wave and ST segment level should probably be additionally analyzed. ECG recordings on patients undergoing similar surgery without ECT and the same post-operative care are also required.

Another future prospect is the evaluation of safety limits of electroporation pulse characteristics for safe application of ECT in vicinity of the heart. The critical distance between location of electroporation pulse delivery and the heart, and the maximal allowed energy and current delivered on the heart muscle to prevent side effects should be determined for different protocols of electroporation pulse delivery used in ECT of deep seated tumors.

In the future, safety limits of electroporation pulse characteristics for safe application of ECT on patients with clinically manifested heart arrhythmias or with implanted pacemaker, which are currently excluded from ECT treatment, should also be estimated.

5.2. Development and evaluation of the algorithm for synchronization of electroporation pulses with electrocardiogram

5.2.1. Performance of the algorithm

- Our algorithm for synchronization of electroporation pulses with electrocardiogram (ECG) enables delivery of electroporation pulses only outside the vulnerable period and prevents pulses from being delivered in case of the appearance of heart arrhythmias, such as atrial and ventricular premature beats [**Paper I**, **Paper V**].
- The algorithm is effective, reliable and appropriate for real-time application [**Paper I**, **Paper V**]. The estimated time reserve for safe electroporation pulse delivery after the

QRS detection and before the onset of vulnerable period for ventricles is approximately 60 ms and is long enough for safe electroporation pulse delivery, even if we want to avoid the vulnerable period of atria as well.

- The algorithm provides effective and reliable synchronization of electroporation pulses with ECG for use in all medical applications that include delivery of high-voltage pulses (like in ECT, gene electrotransfection and irreversible electroporation techniques), regardless of tumor location (even close to the heart) [**Paper I**, **Paper V**].

5.2.2. Performance of our algorithm in comparison with synchronization protocol currently used in clinical electrochemotherapy

- Our algorithm for synchronization of electroporation pulse delivery with ECG is more accurate and safer than the algorithm currently used in clinical ECT [**Paper II**]. In spite of higher complexity and better performance it also does not significantly increase the duration of ECT procedure.
- Synchronization algorithm currently implemented in clinical device for ECT should be improved to prevent electroporation pulse delivery under certain conditions of increased probability for external induction of ventricular fibrillation (such as in case of premature heartbeats) [**Paper II**].

5.2.3. Future prospects for algorithm for synchronization of electroporation pulses with electrocardiogram

The possibilities for future work on synchronization of electroporation pulse delivery with ECG include: implementation of synchronization algorithm in stand-alone device that will enable coupling with currently used medical device for ECT, Cliniporator Vitae, or incorporation of synchronization algorithm in Cliniporator Vitae, and development of the algorithm that will enable safe synchronization of electroporation pulse delivery with ECG also for patients with clinically manifested heart abnormalities, which are currently excluded from ECT treatment.

5.3. Evaluation of effectiveness of clinical electrochemotherapy and its dependence on treatment conditions

5.3.1. Overall effectiveness of electrochemotherapy

- Up-to-date overall effectiveness with complete response rate (CR%) of 59.4% and objective response rate (OR%) of 84.1% for single-session ECT of cutaneous and subcutaneous tumors was determined [**Paper VI**].
- Electroporation pulse delivery significantly potentiates the effectiveness of chemotherapeutic drug alone by more than 50% [**Paper VI**]. CR% of 8.0% and OR% of 19.9%, respectively, for treatment with bleomycin or cisplatin alone were determined.

5.3.2. Evaluation of effectiveness of electrochemotherapy with respect to heterogeneous treatment conditions

- The differences in effectiveness of single-session ECT depend significantly on several treatment conditions, i.e. on chemotherapeutic drug, tumor histotype and tumor size [**Paper VI**, **Paper VI**].
- The effectiveness of ECT of cutaneous and subcutaneous tumors is significantly higher for intratumoral than for intravenous administration of bleomycin [**Paper VI**]. Bleomycin and cisplatin administered intratumorally results in equal effectiveness of ECT [**Paper VI**].
- Non-melanoma tumors respond significantly better to ECT than melanoma tumors [**Paper VI**]. ECT is more effective in sarcoma than in melanoma or carcinoma tumors. Among all types of tumors, basal cell carcinomas have the highest and squamous cell carcinomas the lowest overall CR% and OR% [**Paper VI**].
- Effectiveness of single-session ECT of cutaneous and subcutaneous tumors with maximal diameter equal to or larger than 3 cm is significantly lower than on smaller tumors [**Paper VII**].
- Effectiveness of single-session ECT of cutaneous and subcutaneous tumors decreases progressively with increasing tumor diameter. Tumor size starts to play a significant role in the final treatment outcome for tumors as small as about 2 cm in diameter [**Paper VII**].

5.3.3. Refinement of standard operating procedures

- The results of our systematic review with respect to the effectiveness of clinical ECT should be taken into account in further refinement of ECT protocols on cutaneous and subcutaneous tumors as well as in development of ECT procedures for treating deep seated tumors [**Paper VI**].
- Standard operating procedures for ECT should be refined for the treatment of larger tumors and should include accurate ECT treatment planning or, alternatively, the application of fixed-geometry electrodes with their accurate repositioning in order to overlap the treated volumes, intratumoral and intravenous administration of bleomycin, suggestion for repetitive treatments, and prolonged observation of tumor response [**Paper VII**].

5.3.4. Future prospects on effectiveness of clinical electrochemotherapy

In our future work, the influence of other tumor and treatment parameters (tumor location; amplitude of current, voltage and energy delivered per tumor volume; electrode type; timing of electroporation pulse delivery; median follow-up) on effectiveness of ECT should be evaluated with initiated ad hoc study or when there will be enough published clinical reports that will include details concerning these and also other parameters for individual tumors (such as age and gender of the patient; data about the longest perpendicular diameters of the treated tumor; tumor histotype; drug type, dose and route of administration; protocol of EP pulse delivery; number of EP applications and ECT sessions,…).

6. ORIGINAL CONTRIBUTIONS

Based on results in this doctoral thesis, the following original scientific contributions to the research area were recognized:

6.1. EVALUATION OF SAFETY OF CLINICAL ELECTROCHEMOTHERAPY

In this thesis, safety of clinical electrochemotherapy (ECT) with respect to its possible influence on functioning of the heart was evaluated for the first time. We investigated possible early and late effects of high-voltage electroporation pulses and chemotherapeutic drug on functioning of the heart. The analysis was performed on electrocardiogram (ECG) signals recorded during ECT of cutaneous and subcutaneous tumors [**Paper I**], where the treated region is on the skin and thus located relatively distantly from the heart, and before, during and after ECT of deep seated tumors (i.e. colorectal metastases in liver) [**Paper II**, **Paper III**, **Paper IV**], where the treated region is located in a highly conductive medium and relatively close to the heart. According to the results that show the existence of early and late effects of ECT on functioning of the heart, the importance of synchronization of electroporation pulses with refractory period of the heart cycle was emphasized.

6.2. DEVELOPMENT AND EVALUATION OF THE ALGORITHM FOR SYNCHRONIZATION OF ELECTROPORATION PULSES WITH ELECTROCARDIOGRAM

Currently implemented synchronization protocol in Cliniporator Vitae, the clinical device for ECT of deep seated tumors, provides only basic synchronization capabilities and does not guaranty the delivery of electroporation pulses exclusively outside the vulnerable period of the heart. In this thesis, we developed the synchronization algorithm that delivers electroporation pulses only outside the vulnerable period and prevents pulses from being delivered in case of the appearance of heart arrhythmias, such as atrial and ventricular premature beats [**Paper I, Paper V**]. The algorithm provides effective and reliable synchronization of electroporation pulses with ECG for use in all medical applications that include delivery of high-voltage pulses (like in ECT, gene electrotransfection and irreversible

electroporation techniques), regardless of tumor location (even close to the heart). The algorithm presents an important improvement over currently implemented synchronization protocol in clinical devices for ECT and non-thermal irreversible electroporation [**Paper II**].

6.3. Evaluation of effectiveness of clinical electrochemotherapy and its dependence on treatment conditions

No comprehensive systematic investigation of effectiveness of clinical ECT based on statistical synthesis of tumor response data from different clinical studies has been performed until now. In this thesis, a first such systematic review including meta-analysis technique was performed on data accumulated from all published primary clinical research reports on clinical ECT [**Paper VI**, **Paper VII**]. Overall effectiveness of single-session ECT was estimated and compared to chemotherapy alone. It was shown in this thesis that the differences in effectiveness of single-session ECT depend on several treatment conditions, e.g. on chemotherapeutic drug, tumor histotype and tumor size. The results of our systematic review shed new light on effectiveness of ECT and can be used for prediction of tumor response to ECT with respect to various treatment conditions and should be taken into account in further refinement of ECT protocols on cutaneous and subcutaneous tumors as well as in development of ECT procedures for treating deep seated tumors.

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APPENDIX

PAPER I

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ORIGINAL ARTICLE

The effect of electroporation pulses on functioning of the heart

Barbara Mali · Tomaz Jarm · Selma Corovic · Marija Snezna Paulin-Kosir · Maja Cemazar · Gregor Sersa · Damijan Miklavcic

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Abstract Electrochemotherapy is an effective antitumor treatment currently applied to cutaneous and subcutaneous tumors. Electrochemotherapy of tumors located close to the heart could lead to adverse effects, especially if electroporation pulses were delivered within the vulnerable period of the heart or if they coincided with arrhythmias of some types. We examined the influence of electroporation pulses on functioning of the heart of human patients by analyzing the electrocardiogram. We found no pathological morphological changes in the electrocardiogram; however, we demonstrated a transient RR interval decrease after application of electroporation pulses. Although no adverse effects due to electroporation have been reported so far, the probability for complications could increase in treatment of internal tumors, in tumor ablation by irreversible electroporation, and when using pulses of longer durations. We evaluated the performance of our algorithm for synchronization of electroporation pulse delivery with electrocardiogram. The application of this algorithm in clinical electroporation would increase the level of safety for the patient and suitability of electroporation for use in anatomical locations presently not accessible to existing electroporation devices and electrodes.

Keywords Electrochemotherapy · Electrocardiogram · QRS detection · Synchronization of electroporation pulse delivery with ECG

B. Mali · T. Jarm · S. Corovic · D. Miklavcic (✉)
Laboratory of Biocybernetics, Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, 1000 Ljubljana, Slovenia
e-mail: damijan.miklavcic@fe.uni-lj.si
URL: http://lbk.fe.uni-lj.si/

B. Mali
e-mail: barbara.mali@fe.uni-lj.si

T. Jarm
e-mail: tomaz.jarm@fe.uni-lj.si

S. Corovic
e-mail: selma.corovic@fe.uni-lj.si

M. S. Paulin-Kosir · M. Cemazar · G. Sersa
Institute of Oncology Ljubljana, Zaloska 2, 1000 Ljubljana, Slovenia
e-mail: skosir@onko-i.si

M. Cemazar
e-mail: mcemazar@onko-i.si

G. Sersa
e-mail: gsersa@onko-i.si

1 Introduction

The combined treatment in which delivery of chemotherapeutic drug is followed by application of high-voltage electric pulses locally to the tumor has been termed electrochemotherapy. The effect of local electropermeabilization of the cell membrane (the disruption of the lipid matrix and creation of aqueous pathways [10, 38]), also termed electroporation, transiently enables the entry of anticancer drugs, such as bleomycin or cisplatin, into the cells and hence greater effectiveness of tumor treatment. Electrochemotherapy has been successfully used for treatment of cutaneous and subcutaneous tumors irrespective of their histological origin in different animal tumor models and in humans [19, 36, 51, 52]. In these studies, a typical electrochemotherapy protocol involved eight electroporation pulses (EP pulses) with amplitude of about 1,000 V, duration 100 μs, repetition frequency 1 Hz, and inter-electrode distance 8 mm. However, the protocol involving eight EP

pulses at repetition frequency of 5 kHz has been suggested and is currently replacing the 1-Hz protocol due to a lesser discomfort and pain inflicted to patients [31, 57]. Electrodes of three different configurations can be used for EP pulse delivery during electrochemotherapy. EP pulses applied by plate electrodes are used in case of superficial tumor nodules whereas EP pulses to deeper-seated tumors (subcutaneous nodules) are applied using needle row array electrodes (eight needle electrodes arranged in two rows) or needle hexagonal array electrodes (six hexagonally arranged electrodes with the seventh electrode in the centre) [31]. The number of applied EP pulses and pulse repetition frequency depend on the electrode type and define the duration of electroporation, which is 1.6 ms for plate and needle row array electrodes [31] and approximately 200 ms for needle hexagonal array electrodes [31, 45]. New protocols for delivery of EP pulses are either already in use or are being developed. For gene electrotransfer three different EP pulse protocols are in use: short high-voltage EP pulses, EP pulses of a much longer duration (in the order of milliseconds), or combination of short high-voltage EP pulses with very long low-voltage electrophoretic pulses (amplitude 50–100 V, duration 100 ms) [8, 18, 20, 40, 48]. Tumor ablation by irreversible electroporation is another recently developed application, where EP pulses with larger amplitudes (up to 3,000 V) and longer durations (up to 24 ms) are delivered [3, 14, 28, 35]. New applications using endoscopic or surgical means to access internal tumors are also being developed [21].

Electrochemotherapy is reported as an efficient and safe method. No adverse effects have been reported so far. Electrochemotherapy causes only minor side effects in the patients such as the transient lesions in areas in direct contact with the electrodes [37] and acute localized pain due to contraction of muscles in vicinity of the electrodes [36, 57]. The induced contraction could present a problem if provoked in the heart muscle [46]. There is very little chance that currently used electroporation protocols could interfere with functioning of the heart since there is no such practical evidence. However, this issue has not been systematically investigated yet. Given the increasing need for palliative treatment of internal tumors, the possibility of EP pulses interfering with functioning of the heart is emerging for tumors located close to the heart muscle. Among possible irregularities of functioning of the heart that the application of EP pulses could induce (e.g., atrial and ventricular flutter and fibrillation, premature heartbeats), the most dangerous is ventricular fibrillation [46]. Fibrillation can be induced if electrical stimulus is delivered during late atrial or ventricular systole, during the so-called vulnerable period of the heart [25, 46, 55] (Fig. 1). For ventricular myocardium, the vulnerable period coincides with the middle and terminal phases of the T wave [46], but higher shock strengths cause the vulnerable period to occur

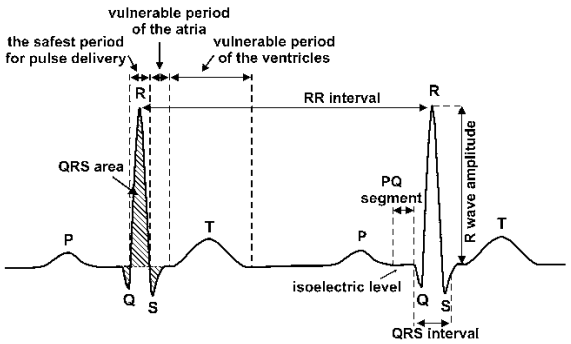

Fig. 1 The vulnerable period and characteristics of a typical heartbeat

several milliseconds earlier in the heartbeat [27]; therefore, the whole T wave can be considered to be within the vulnerable period of the ventricles. For the atria, the vulnerable period is somewhere in the S wave [46]. Externally applied electric pulses delivered outside the vulnerable period have extremely low probability of inducing ventricular fibrillation [46]. According to this fact the synchronization of EP pulse delivery with electrocardiogram (ECG) would increase safety of the patient. The likelihood of electroporation to influence functioning of the heart depends also on applied pulse voltage, duration, number and repetition frequency of EP pulses, and electric current pathway [46].

Although fibrillation can occur in normal and healthy hearts, it is more likely in hearts with structural or functional abnormalities [11]. Abnormalities of the heart rhythm (arrhythmias) are indicated by significant deviation of RR interval from its normal value [12, 46]. During some arrhythmias the heart becomes more susceptible to external stimuli due to a decreased threshold level for fibrillation. Therefore EP pulses coinciding with some arrhythmias could elicit fibrillation. This potential danger is most significant after premature heartbeat, the extrasystole [46].

The main purpose of this study was therefore to investigate the possible effects of EP pulses on functioning of the heart and to address the relevance of synchronization of EP pulse delivery with ECG. In this context we also evaluated the performance of our previously developed algorithm for QRS detection and synchronization of EP pulse delivery with ECG [30].

2 Methods and materials

2.1 Patients and electrochemotherapy

Fourteen human patients were included in this study. Before electrochemotherapy treatment a signed consent was obtained from each patient. Patients were treated

according to the electrochemotherapy protocols as described by Marty et al. [31] with the addition of ECG monitoring. Electrochemotherapy drugs (cisplatin or bleomycin) were administered locally to tumors. EP pulses were generated by the electric pulse generator Cliniporator™ (IGEA S.R.L., Carpi, Italy). Altogether 93 applications of EP pulses were performed. Main characteristics of the patients, tumors and electrochemotherapy are presented in Table 1.

We recorded 16 ECG signals on 14 patients during electrochemotherapy at the Institute of Oncology in Ljubljana. Two patients were treated twice. Thus ECG signals number 1 and 2 belong to the same person as well as signals number 5 and 6 (Table 1). ECG signals were acquired at sampling frequency of 250 Hz using a BIOPAC data acquisition and measurement system (BIOPAC Systems, Inc., USA). To enable early detection of QRS complex we required an ECG lead, which results in a distinctive ascendant QR junction, high R wave amplitude and high dynamics within the QRS complex in comparison to other parts of the ECG signal. Typical standard ECG leads fulfilling these requirements include the chest lead V_4 and standard limb leads I, II and III. We recorded ECG signals from leads I and III by placing the electrodes (disposable soft cloth ECG electrodes, diameter 6 cm, 3M™ Red Dot™) on wrists and ankles and computed the third limb lead II by summing the leads I and III. The lead with best dynamic characteristics was selected for the analysis individually for each patient (see Table 5).

2.2 Analysis of electrocardiograms

The primary analysis of ECG signals recorded during electrochemotherapy was made by using QRS detection algorithm based on the analysis of a single lead ECG, which enables EP pulse delivery prior to the vulnerable period of the heart [30]. This algorithm for synchronization of EP pulse delivery with ECG was developed and evaluated using records of the Long-term ST database (LTST DB database) [23] and was written in ANSI C programming language. The algorithm is described in detail elsewhere [30]. Briefly, it consists of two major components (the detection phase and the decision-making phase), which are preceded by the learning phase during which architecture parameters are estimated from the ECG signal. The detection phase is based on consideration of several ECG signal features: the QR interval, the R wave amplitude and the RR interval (see Fig. 1), in order to achieve a reliable QRS detector performance and to assure clear distinction between normal and abnormal individual heartbeats. For implementation of such a detector the peaks of Q and R waves and the isoelectric level are extracted from the ECG signal. During the decision-making phase, a decision is made whether the EP pulse can be delivered or not based on evaluating deviations of R wave amplitude and RR interval of individual heartbeat from moving average values of these two parameters.

Further analysis of ECG signals recorded during electrochemotherapy was performed to estimate the effect of EP pulse delivery on ECG. For this purpose the peaks of S waves and ends of QRS complexes were determined using program routines written in Matlab. Since the longest normal duration of the QRS complex is 120 ms [22] and the R peak is located approximately at the centre of the QRS complex, it is reasonable to expect the S peak within an interval of 60-ms after the R peak. The algorithm calculates the first derivative of the ECG signal in this 60 ms interval and looks for the first occurrence of three successive samples with negative first derivative followed by a sample with nonnegative derivative. The S peak is assigned to the third of these four samples. Next, the algorithm searches for the flattest part of the ST segment in order to determine the end of the QRS complex. For this purpose an interval of 40 ms after the S peak is analyzed. The average value of five successive samples from this interval having the minimal total deviation from their average value is taken as the flattest part of the ST segment and the middle sample is considered as the end of QRS complex, i.e., the end of QRS interval. After this, the area under the QRS complex is estimated. A similar routine is used for localization of the end of T wave except that an interval of 130 ms after the T peak is analyzed; the flattest part is searched for and the middle sample of this flattest part is considered as the end of T wave. The corrected QT interval (QTc interval) is calculated as the QT interval divided by the square root of the corresponding RR interval.

For evaluation of the effects of EP pulse delivery on functioning of the heart we calculated the average values of RR interval, QRS interval, QTc interval, QRS area and R wave amplitude before and after the application of EP pulses. The length of the averaging interval (8.5 s) was chosen based on the minimum interval between two successive applications of EP pulses, which was 8.5 s.

Testing and evaluation of this newly developed part of the algorithm was performed by manual verification of automatically defined locations of S peak, T peak, the end of T wave, and duration of QRS interval on randomly selected sequences of ECG signals included in our study. In addition, since this was the first application of the newly developed part algorithm, we also manually verified the results of the algorithm on 8.5 s-long segments of ECG signals before and after all 93 applications of EP pulses.

All ECG signals were manually examined by two medical doctors (including a cardiologist), who classified all abnormal heartbeats present in the signals. Other heartbeats were considered as normal. They found no evidence of significant

Table 1 Main characteristics of the patients, tumors and electrochemotherapy pulses for individual ECG signal

Signal number	Patient				Tumor			Electrochemotherapy			
	Sex	Age	BMI	Pre-existing cardiac conditions	Type	Number of nodules	Location of nodules	Electrode type	Number of applications	Voltage (V)	Drug
1	F	78	35.3	None	Malignant melanoma	1	EX: left ankle	Hexagonal[a]	1	730	Cisplatin
2	F	78	35.3	None	Malignant melanoma	1	EX: left lower leg	Plate (6 mm)	1	680	Cisplatin
3	F	71	20.5	Mitral valve prolapse	Infiltrating lobular cancer	1	TR: parasternal right	Plate (8 mm)	4	960	Cisplatin
4	M	75	24.3	Arterial hypertension	Malignant melanoma	1	TR: lower abdomen	Plate (6 mm)	3	680	Bleomycin
5	F	60	24.8	Arterial hypertension	Malignant melanoma	5	EX: left upper leg	Plate (8 mm)	6	960	Bleomycin
6	F	60	24.8	Arterial hypertension	Malignant melanoma	1	EX: under left knee	Plate (8 mm)	1	960	Bleomycin
						5	EX: left upper leg	Plate (8 mm)	5	960	
7	F	48	21.7	None	Malignant melanoma	1	TR: thorax, right side	Plate (8 mm)	4	960	Bleomycin
						1	TR: back, right side	Plate (8 mm)	7	960	
8	F	68	23.2	None	Malignant melanoma	1	EX: left instep	Hexagonal[a]	3	730	Bleomycin
9	F	92	20.8	Arterial hypertension	Malignant melanoma	10	EX: left lower leg	Plate (6 mm)	10	680	Cisplatin
10	F	73	27.3	Arterial hypertension	Invasive ductal carcinoma	11	TR: thorax	Plate (6 mm)	11	680	Bleomycin
11	F	72	20.0	None	Malignant melanoma	1	EX: under right knee	Plate (6 mm)	1	680	Cisplatin
12	M	67	21.5	None	Malignant melanoma	1	TR: thorax left side	Plate (6 mm)	2	680	Cisplatin
						1	TR: thorax right	Plate (6 mm)	2	680	
						1	EX: left upper arm	Plate (6 mm)	4	680	
13	F	80	23.5	None	Malignant melanoma	3	EX: left lower leg	Plate (6 mm)	3	680	Cisplatin
						1	EX: left upper leg	Plate (6 mm)	1	680	
14	F	79	28.6	Arterial hypertension	Malignant melanoma	1	EX: under right knee	Plate (6 mm)	6	680	Cisplatin
						1	EX: right ankle-back	Plate (6 mm)	4	680	
15	F	81	NA	None	Malignant melanoma	6	EX: left lower leg	Plate (6 mm)	9	680	Cisplatin
16	F	52	32.4	None	Sarcoma	1	TR: left hip	Plate (6 mm)	5	680	Cisplatin

BMI body mass index, *NA* not available, *EX* location on extremities, *TR* location on trunk

[a] Penetration depth 3 and 2 mm, respectively

long-lasting heart arrhythmias (e.g., bradycardia, tachycardia). For evaluation of QRS complex detection, we calculated the following scores for each record: N_d, TP, FN and FP (for definitions see Table 5). Based on these scores obtained with a beat-by-beat comparison of the results of our algorithm [30] with the medical expert-defined annotations of the heartbeats, we calculated standard performance measures of the algorithm: the sensitivity (Se), the positive predictivity ($+P$) and the detection error rate (DER) for QRS detection (Eqs. 1–3, respectively). The performance measures for an ideal QRS detector would be $Se = 100\%$, $+P = 100\%$ and $DER = 0\%$.

$$Se(\%) = \frac{TP}{N_d} \times 100 \quad (1)$$

$$+P(\%) = \frac{TP}{TP + FP} \times 100 \quad (2)$$

$$DER(\%) = \frac{FP + FN}{N_d} \times 100 \quad (3)$$

For evaluation of EP pulse delivery we calculated the following scores for each record: N_p, TP_p, FN_p and FP_p (for definitions see Table 5). Based on these scores and in the absence of any standard performance metrics for EP pulse delivery, we calculated the performance measures analogous to QRS detection metrics: the sensitivity (Se_p), the positive predictivity ($+P_p$) and the delivery error rate (DER_p) for EP pulses. The performance measures for an ideal algorithm for EP pulse delivery would be $Se_p = 100\%$, $+P_p = 100\%$ and $DER_p = 0\%$.

The performance of our algorithm for QRS detection and EP pulse delivery has previously been evaluated on ECG signals from a standard LTST DB database [30]. The results were: $Se = 99.4\%$, $+P = 100.0\%$, $DER = 0.6\%$, $Se_p = 91.8\%$, $+P_p = 100.0\%$ and $DER_p = 8.3\%$ (median values).

2.3 Numerical modeling

We performed numerical calculations of electric field and current distribution for tissue models. The geometry of models and electrode configurations are shown in Fig. 2. The modeled conditions (needle row array, needle hexagonal array and plate electrode configurations and voltages applied) were the same as actually used in clinical electrochemotherapy (see Tables 3 and 4 for details). The modeled tissues (the target tumor tissue and the surrounding healthy tissue) are treated as isotropic materials with ohmic behavior (only conductivity of the tissues was taken into account). The assigned conductivity values were set to be 0.4 S/m for the tumor and 0.2 S/m for the healthy tissue according to previous measurements of tumor and tissue conductivity [34], models of subcutaneous tumor and skin electropermeabilization [43], a 3D finite element model of thorax, where the sensitivity of defibrillation parameters to the variations in model inhomogeneity and approximation of skeletal muscle anisotropy was examined for different paddle placements [9], and average conductivity of tissues composing the thorax [26]. The conductivity of cardiac muscle was reported to be in the range between 0.17 and 0.25 S/m [9, 26]. The assigned conductivity values for target tumor tissue (0.4 S/m) and the surrounding healthy tissue (0.2 S/m) describe the conductivity at the end of the electropermeabilization process, thus incorporating the changes to tissue conductivity due to exposure to external electric pulses.

The critical depth for electric field of 200 and 450 V/cm (value for reversible and irreversible electroporation of the muscle, respectively [43]), by solving the Laplace equation, and the critical depth for current of 100 mA (threshold for ventricular fibrillation for 500 μs-long electrical stimulus [46]), were estimated by means of finite element method using COMSOL Multiphysics 3.3 software package (COMSOL AB, Sweden). Of the total electric current flowing through the tissue during the EP pulse delivery, no more than 100 mA (the threshold value for fibrillation) is allowed to flow through the heart. Therefore we defined the critical depth as a distance from the surface of the body (at the site of EP delivery) below which the total electric current flowing is equal to this threshold value. This is a very conservative approach in which it is assumed that the entire current flowing below the critical depth actually passes through the heart. The validity of the model is further discussed in the Sect. 4.1.

2.4 Statistical analysis

The performance of the algorithm and average values of heartbeat parameters were compared using either the Mann–Whitney Rank Sum or Wilcoxon Signed Rank test. In all tests, a p value of less than 0.05 was considered as indication of statistically significant difference. The statistical analysis was performed using SigmaStat 3.1 software package. Since the data were not normally distributed, we give statistical summary of the results using both the mean/standard deviation and the median/quartile values. However, when we say "on average" in the text we are referring to median values, which are more representative of the middle of the sample and population than the mean values.

3 Results

3.1 The effect of electroporation pulse delivery on electrocardiogram

Our program-based analysis of heartbeat characteristics (RR interval, QRS interval, QTc interval, R wave and QRS

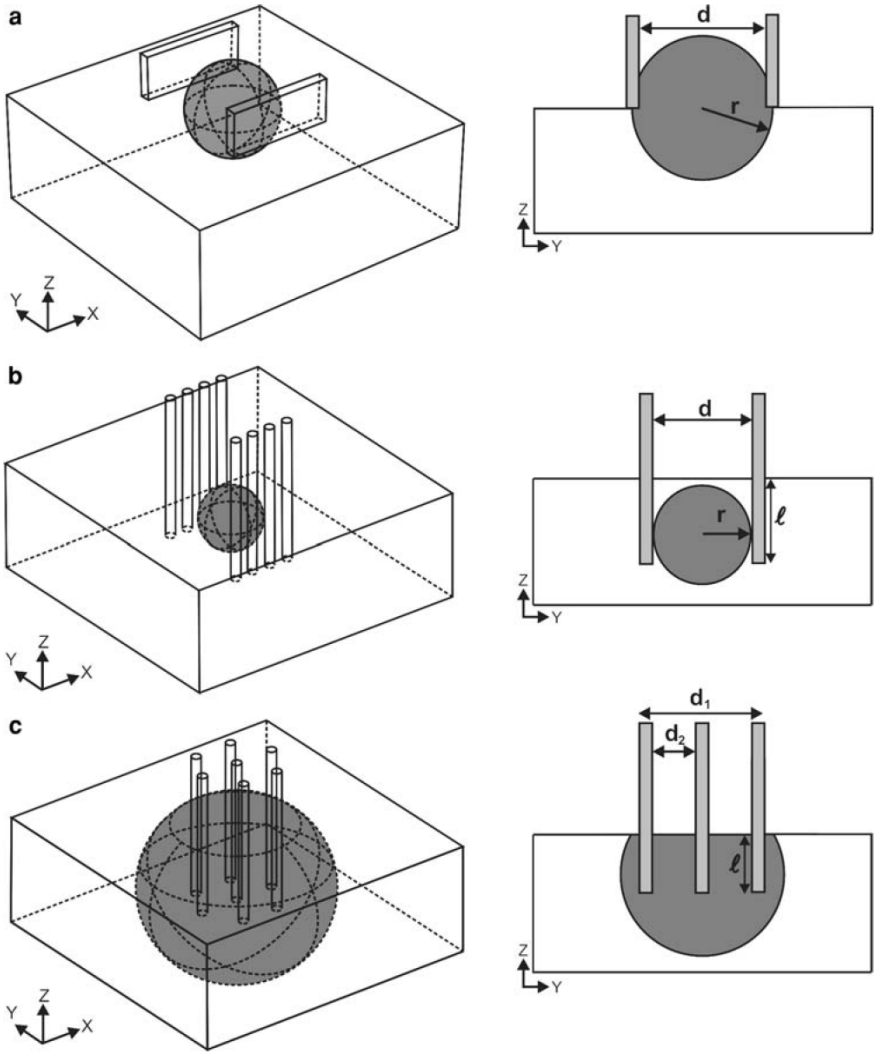

Fig. 2 The geometry of tissue models with tumor for: **a** plate electrodes (length 7 mm, thickness 0.7 mm, distance between the electrodes d is 4, 6 or 8 mm, tumor diameter is 1 mm larger than distance between the electrodes); **b** needle row array electrodes (diameter 0.7 mm for each needle, distance between two rows of electrodes $d = 4$ mm, tumor radius $r = 4$ mm, tumor location 0.5 mm under the skin surface); **c** needle hexagonal array electrodes (diameter 0.7 mm for each needle, distance between two electrodes 8 mm, tumor radius $r = 15$ mm)

area) revealed no pathological morphological changes caused by EP pulses in patients subjected to electrochemotherapy. This finding was confirmed independently by two medical doctors. The significant change, however, was detected in RR and QRS interval duration after each application of EP pulses (see Table 2).

The medical doctors involved in the study confirmed that EP pulses induced no heart arrhythmias. Moreover, additional premature heartbeats were not triggered by EP pulses in the cases where premature heartbeats were present in ECG signal before the application of EP pulses.

The results of modeling the distribution of electric field and current in tissue models are presented in Tables 3 and 4. It can be seen that in the worst-case scenario (needle hexagonal array electrodes, 10 mm depth of insertion) the critical depth for current of 100 mA is 4.10 cm. The largest critical depths for reversible and irreversible electroporation are 1.30 and 1.07 cm, respectively, for needle row array electrodes at insertion depth 10 mm.

Table 2 The change in heartbeat parameters after EP pulse delivery

Evaluated parameters	Median change	Percentile		Statistical significance (p)
		25%	75%	
RR interval (ms)	−5.43	−19.60	7.72	0.006
QRS interval (ms)	−1.25	−9.13	4.13	0.042
R wave amplitude (mV)	6.46	−28.50	35.20	0.414
QRS area (mV ms)	515	−671	2,010	0.091
QTc interval (ms)	1.89	−9.73	11.40	0.380

Wilcoxon Signed Rank test, $n = 93$

Table 3 Calculated critical depths for electric field and current in different plate electrode configurations

Distance between electrodes (d) (mm)	Tumor radius (r) (mm)	Applied voltage (V)	Critical depth for 100 mA (cm)	Critical depth for 200 V/cm (cm)	Critical depth for 450 V/cm (cm)
4	2.5	520	1.00	0.31	0.18
6	3.5	780	1.55	0.43	0.22
8	4.5	1,000	2.38	0.51	0.30

Table 4 Calculated critical depths for electric field and current in needle row array and needle hexagonal array electrode configurations

Depth of insertion (l) (mm)	Needle row array electrodes (400 V, tumor radius $r = 2$ mm)			Needle hexagonal array electrodes (730 V, tumor radius $r = 15$ mm)		
	Critical depth for 100 mA (cm)	Critical depth for 200 V/cm (cm)	Critical depth for 450 V/cm (cm)	Critical depth for 100 mA (cm)	Critical depth for 200 V/cm (cm)	Critical depth for 450 V/cm (cm)
2	1.55	0.43	0.26	2.23	0.43	–
4	2.37	0.68	0.46	2.65	0.64	0.30
6	2.94	0.90	0.70	3.20	0.87	0.54
8	3.40	1.10	0.90	3.69	1.04	0.70
10	3.79	1.30	1.07	4.10	1.25	0.93

3.2 The algorithm for synchronization of electroporation pulse delivery with electrocardiogram

The performance of the algorithm for QRS detection and synchronization of EP pulse delivery with ECG is summarized in Table 5. On average, the algorithm correctly detected 99.2% of all QRS complexes. The total number of erroneously detected QRS complexes was 15. On average, the algorithm would correctly deliver EP pulses in 94.6% of normal QRS complexes. The average positive predictivity for EP pulses ($+P_p$) was 100.0% and thus ideal.

A comparison of performance between 16 ECG signals recorded during electrochemotherapy and 42 ECG signals from the LTST DB database [30] was performed. The results showed that there is not a statistically significant difference in the median values between all compared performance measures (Se, $+P$, DER, Se_p, DER_p) ($0.142 < p < 0.924$) except for the positive predictivity for EP pulse delivery ($+P_p$) ($p = 0.026$, Mann–Whitney Rank Sum test). This performance measure was significantly better for ECG signals recorded during electrochemotherapy than for ECG signals from LTST DB database.

4 Discussion

4.1 The effect of electroporation pulse delivery on electrocardiograms

We found no heart arrhythmias or other pathological morphological changes of heartbeat as a consequence of applied EP pulses. No additional premature heartbeats were triggered even in cases when these were present in ECG signal before the first application of EP pulses (signals number 11, 12 and 16). This is in agreement with the results of the work by Al-Khadra et al. [2] that showed no arrhythmias in association with electroporation applied directly on the heart. According to the heart strength-duration curve a very large current would be required to cause a single premature heartbeat [7, 15, 46] for very short EP pulse duration (the microsecond range). Since no additional premature heartbeats were detected, it is highly improbable that EP pulses alone could create the inhomogeneity (altered states of depolarization–repolarization), which is a requisite for onset of fibrillation.

The computer-based analysis demonstrated no significant statistical change in the QTc interval but a significant statistical decrease in the RR and QRS interval after each application of EP pulses (Table 2). This transient effect disappeared within 10 s after each application of EP pulses. The RR and QRS intervals are tightly correlated because they are both dependent on the heartbeat frequency [46]. A significant change in QT interval is one of the most important indicators of arrhythmias [4]. However, its value is also dependent on the heart rate (the faster the heart rate, the shorter the QT interval) and has to be adjusted to aid interpretation. For this reason the QTc interval is used in practice. A significant change in the QTc interval would indicate a clinically relevant effect of electrochemotherapy. However, no such effect was observed in our study (1.89 ms median change of QTc interval after application of EP pulses, see Table 2).

Table 5 ECG signals, ECG lead used, number of heartbeats of particular type, the results of QRS detection and the results of EP pulse delivery

Signal number	Lead name	P	N_d	TP	FN	FP	Se (%)	$+P$ (%)	DER (%)	N_p	TP_p	FN_p	FP_p	Se_p (%)	$+P_p$ (%)	DER_p (%)
1	I	0	461	460	1	1	99.8	99.8	0.4	461	452	9	0	98.0	100.0	2.0
2	I	0	609	607	2	0	99.7	100.0	0.3	609	602	7	0	98.9	100.0	1.1
3#	I	0	462	455	7	2	98.5	99.6	1.9	462	415	47	0	89.8	100.0	10.2
4	I	0	354	351	3	2	99.2	99.4	1.4	354	334	20	0	94.4	100.0	5.7
5	I	0	1,716	1,714	2	0	99.9	100.0	0.1	1,716	1,702	14	0	99.2	100.0	0.8
6	II	0	1,822	1,819	3	0	99.8	100.0	0.2	1,822	1,809	13	0	99.3	100.0	0.7
7	II	0	1,635	1,622	13	1	99.2	99.9	0.9	1,635	1,548	87	0	94.7	100.0	5.3
8	I	0	415	415	0	0	100.0	100.0	0.0	415	412	3	0	99.3	100.0	0.7
9	I	1	1,405	1,397	8	2	99.4	99.9	0.7	1,404	1,359	45	0	96.8	100.0	3.2
10#	I	1	1,264	1,260	4	0	99.7	100.0	0.3	1,263	991	272	0	78.5	100.0	21.5
11#	III	131	795	664	131	0	83.5	100.0	16.5	664	422	242	0	63.6	100.0	36.4
12	II	17	1,348	1,330	18	0	98.7	100.0	1.3	1,331	1,307	24	0	98.2	100.0	1.8
13#	I	1	678	666	12	0	98.2	100.0	1.8	677	589	55	0	87.0	100.0	8.1
14#	I	0	1,009	1,001	8	6	99.2	99.4	1.4	1,009	540	469	0	53.5	100.0	46.5
15	I	8	798	784	14	0	98.2	100.0	1.8	790	746	44	0	94.4	100.0	5.6
16	I	18	1,384	1,358	26	1	98.1	99.9	2.0	1,366	1,291	75	0	94.5	100.0	5.5
Total	–	177	16,155	15,903	252	15	–	–	–	15,978	14,519	1,426	0	–	–	–
Min	–	0	354	351	0	0	83.5	99.4	0.0	354	334	3	0	53.5	100.0	0.7
25%	–	0	536	534	3	0	98.4	99.8	0.3	536	437	14	0	88.4	100.0	1.5
Median	–	0	904	893	8	0	99.2	100.0	1.1	900	674	45	0	94.6	100.0	5.4
75%	–	5	1,395	1,378	14	2	99.7	100.0	1.8	1,385	1,333	81	0	98.5	100.0	9.1
Max	–	131	1,822	1,819	131	6	100.0	100.0	16.5	1,822	1,809	469	0	99.3	100.0	46.5
Mean	–	11	1,010	994	16	1	98.2	99.9	1.9	999	908	89	0	90.0	100.0	9.7
St. dev.	–	33	501	504	32	2	4.0	0.2	3.9	505	516	129	0	13.6	0.0	13.6

P premature heartbeats of ventricular, supraventricular or ectopic origin; N_d 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); FP false positive for QRS detection (the number of false QRS detections); Se sensitivity for QRS detection; $+P$ positive predictivity for QRS detection; DER detection error rate for QRS detection; N_p total number of normal QRS complexes (total number of possible delivered EP pulses), the sum of TP_p and FN_p; TP_p true positive for EP pulses (the number of EP pulses delivered at correctly detected normal QRS complexes); FN_p false negative for EP pulses (the number of correctly detected normal QRS complexes, where no EP pulse was delivered); FP_p false positive for EP pulses (the number of EP pulses delivered in the absence of correctly detected normal QRS complexes); Se_p sensitivity for EP pulses; $+P_p$ positive predictivity for EP pulses; DER_p delivery error rate for EP pulses

ECG signals with relatively poor values of performance metrics

Several studies suggested that there is a link between negative emotions (e.g., anxiety) and the oscillations of RR interval [1, 16, 24, 32, 53, 56]. The most frequently reported symptoms in panic attacks, which are characterized by episodes of intense anxiety, are heart pounding and tachycardia [16, 32]. Another possible reason for RR interval decrease is intrinsic sympathetic activation of the nervous system, occurring in response to stress, exercise, or heart disease [1, 53]. The applications of electrochemotherapy to internal tumors could also directly affect the cardiac tissue if tumors were located close to the heart muscle. However, this effect is highly unlikely for current applications of electrochemotherapy because of relatively large distance between treated tumors and the heart (at least several centimeters) and due to small inter-electrode distances (8 mm or less). This assumption is further supported by the calculated critical depths for electric current threshold values discussed later in the text. Therefore, we suggest that the observed changes in RR interval can be largely if not completely attributed to anxiety and stress of the patient undergoing electrochemotherapy.

In our study almost one third (30.5%) of the 93 non-synchronized EP applications were delivered within the vulnerable period, which is in accordance with the fact that the duration of the vulnerable period is around one third of the duration of heart cycle [46]. The study of occurrence of ventricular fibrillation after atrial cardioversion performed with transthoracic electrodes pointed out that delivery of

electric pulse, which was not synchronized with the R wave consistently resulted in ventricular fibrillation if the pulse was delivered within the T wave, the vulnerable period of the ventricles [5]. The probability for ventricular fibrillation was decreased but not eliminated with the arrival of synchronized defibrillators for cardioversion [5]. A high percentage of EP pulses delivered during the vulnerable period in our study, when EP application is not synchronized with ECG, underlines the importance of synchronization.

The values of electric field and current in the heart muscle during electroporation are important for evaluation of the danger for inducing ventricular fibrillation. For tissue models (Fig. 2) we estimated critical depths by calculating threshold value of electric field for reversible and irreversible electroporation of the muscle (200 and 450 V/cm, respectively [43]), and threshold value of current for ventricular fibrillation (100 mA for 500 μs-long stimulus [46]) (see Tables 3 and 4). In the study by Galvão et al. [17] they showed that the threshold current that stimulates the heart strongly depends on the age of the animal (i.e., old animals have lower threshold levels). Due to the relatively old patients included in our study (median value 70.8 years) we can therefore assume that the current threshold value is lowered. On the other hand, the EP pulses used in electrochemotherapy are much shorter (100 μs) and therefore the threshold value should in theory be approximately five times greater than for the 500 μs-long stimulus [15, 46]. Furthermore, amplitude threshold for fibrillation for pulsed direct currents is considerably higher than for alternating currents [46]. Currently we have no conclusive information regarding the influence of the repetition frequency of pulsed direct currents on the threshold for fibrillation. Therefore we adopted the threshold value of 100 mA as an estimate of true threshold value. For the needle row array and needle hexagonal array electrodes the critical depth depends on the depth of insertion. The results showed that for the plate electrodes with 8 mm distance between the electrodes the critical depth for threshold current was 2.38 cm (see Table 3). For needle row array and needle hexagonal array electrodes at depth of insertion of 10 mm, the critical depths for threshold current were 3.79 and 4.10 cm, respectively (see Table 4). The critical depth for threshold current for plate electrodes is smaller in comparison to the needle row array or needle hexagonal array electrodes because most of the voltage drop occurs on the skin [33].

With electrodes positioned distantly from the heart (e.g., on a single limb), as in the majority of EP pulse delivery cases (see Table 1), the current traversing the heart is negligible and therefore a smaller risk of accidental cardiac stimulation exists [44]. EP pulses frequently provoke strong and painful muscle contractions [57]. In contrast to the heart muscle, the motor neurons innervating the skeletal muscles, which are located in relative proximity to the electrodes, are always stimulated by EP pulses.

In electrochemotherapy applications included in our study, plate electrodes with distances between the electrodes of 6 and 8 mm and needle hexagonal array electrodes with small depths of insertion (2 or 3 mm) were used (see Table 1). The majority of the EP pulses were delivered on extremities and even when delivered on the trunk they were delivered distantly from the heart. However, two applications of EP pulses were delivered on the chest relatively close to the heart (distance approximately 5 cm) with plate electrodes. Since the heart lies at least 3 cm beneath the skin surface [54], for the applications involved in our study the results of modeling indicated that it is highly unlikely that EP pulses even when applied on the trunk directly above the heart could affect functioning of the heart (critical depths 2.23 and 2.38 cm for needle hexagonal array and plate electrodes, respectively). Furthermore, the most vulnerable part of the heart, the apex, lies behind the breast and is thus additionally protected from the external stimulation by breast tissue. Additionally, if the electrodes were located above sternum or above a rib, the risk of affecting the heart would be further reduced due to low conductivity of bones (range from 0.01 to 0.06 S/m) [34] and larger dimension of sternum and ribs in comparison to the distance between the electrodes. However, if the electrodes were not located directly above the sternum or a rib, the critical depths would be larger due to higher conductivity of the underlying tissues. In this case according to the results of modeling there exists a theoretical chance to affect the functioning of the heart in case of deep insertion of either needle hexagonal or row array electrodes (approximately 4 cm for both types of electrodes at insertion depth of 10 mm). This should be considered in future applications of electrochemotherapy.

For the modeled tissues only the conductivity was taken into account. The capacitive behavior of the tissues was neglected since the transient effects are present only during the charging time of the cell membrane, which lasts around 1 μs [39]. The membrane charging time is much shorter compared to the EP pulse duration used in electrochemotherapy. It was also shown in several studies that static analysis of the electric field distribution during electroporation without taking into account the transient effects are adequate [43, 49, 50], since after the transient effect the tissue exhibits only ohmic behavior [13]. Using current-voltage measurements on cells in vitro [42] or tissues in vivo [13] it was shown that electroporation occurs after the transient time and that the dynamic behavior at the start of the pulses (that includes capacitive behavior of the tissue) is not crucial for the process of electroporation. On the other hand, this transient effect induces a rapid initial current increase followed by an exponential decrease and a

constant level. The applied current during electrochemotherapy is limited to 16 A. Since the transient effect lasts only 1 μs and according to the heart strength-duration curve it is very unlikely that the transient part of an EP pulse could induce ventricular fibrillation [7, 15, 46].

Since the electrode dimensions (electrode–tissue contact surface area) and distance between electrodes used in electrochemotherapy for tumors analyzed in our study are significantly smaller compared to the electrode dimensions and positions used for cardiac defibrillation, we can assume that the differences in thorax tissues conductivities should not change the electric field and current distribution calculated with our numerical models. Therefore, based on the previous studies [9, 26, 34, 43] we can conclude that our numerical models with chosen electrical properties can be used for the evaluation (rough estimate) of critical electric field and current for protruding cutaneous tumors or subcutaneous tumors, which are located immediately under the skin surface (0.5 mm under the skin surface in our study). However, for more deeply located tumors the exact conductivities of all tissues should be incorporated in the numerical model of the thorax.

Recently a new method of local and drug-free tissue ablation called irreversible electroporation has been developed for clinical use as a promising approach to solid tumor therapy [3, 14, 35], prostate ablation [41] and cardiac tissue ablation [28]. In these studies EP pulses with larger amplitudes (up to 3,000 V [11]) and longer durations (range from 100 to 24 ms) are used. The application of EP pulses during irreversible electroporation is therefore more likely to influence functioning of the heart than EP pulses usually applied in electrochemotherapy. However, the results of a recent study by Lavee et al. [28] using irreversible electroporation for epicardial atrial ablation for the treatment of atrial fibrillation showed that ablation pulses (amplitudes of 1,500–2,000 V, duration 100 μs, frequency 5 Hz) caused no permanent arrhythmia or any other rhythm disturbance apart from the rapid atrial pacing during the pulse sequence application. The immediate resumption of sinus rhythm following the ablation was recorded. Similarly, the results of study by Al-Khadra et al. [2] demonstrated lack of any evidence of spontaneous arrhythmias (reentrant or focal) associated with electroporation of the endocardium or the papillary muscles. In their study they presented experimental evidence suggesting that electroporation might even transiently reduce myocardial vulnerability to arrhythmias. But on the other hand, they pointed out that electric pulses of high energy are known to produce a permanent damage, perhaps associated with electroporation. This effect of electroporation may provide a substrate for arrhythmogenesis [2]. Our results of modeling showed that the critical depths for irreversible electroporation of the muscle/heart (450 V/cm) are 1.07 and 0.93 cm for needle row array and needle hexagonal array electrodes, respectively, at insertion depth 10 mm (Table 3). The critical depth for the plate electrodes is smaller (0.30 cm for 8 mm distance between the electrodes). Since the heart lies at least 3 cm beneath the skin surface [54], for the applications involved in our study the results of modeling indicated that it is impossible to affect functioning of the heart by irreversible electroporation even when EP pulses were applied on the trunk directly above the heart. That would not be the case for electrochemotherapy treatment of internal tumors located close to the heart muscle.

Considering these facts, it is nevertheless advisable to incorporate synchronization of EP pulse delivery with ECG in medical equipment for electroporation in order to maximize safety of the patients especially in future clinical applications. Synchronization of EP pulse delivery with the refractory period of the cardiac cycle is always advisable whenever there is a possibility of EP pulses influencing the functioning of the heart.

4.2 The algorithm for synchronization of electroporation pulse delivery with electrocardiogram

The algorithm for QRS detection and EP pulse delivery reliably detected QRS complexes in all signals recorded during electrochemotherapy (see Table 5). The algorithm would allow for EP pulse delivery only for correctly identified heartbeats with no abnormalities. The performance of our algorithm for QRS detection is similar to that of some other detectors with comparably simple algorithms [6, 47]. The algorithm performed poorly (large *DER*, marked with # in Table 5) for ECG signals with either very unstable R wave amplitudes or RR intervals, or heavy contamination with high-frequency noise with amplitudes similar to R wave, or presence of premature heartbeats not satisfying the dynamic requirements within the QRS complex [12, 29]. In total we found 15 false positive detections (*FP*), which were caused by the occurrence of transient noise having the morphology and the time of appearance so similar to a normal QRS complex that the algorithm could not distinguish them. Many of the false negative detections (*FN*) were due to our self-imposed strict requirements for as few as possible false positive detections (*FP*).

The most appropriate time for EP pulse delivery is before the onset of the vulnerable period since the vulnerable period can sometimes be prolonged (e.g., after premature heartbeat) [46]. Thus delivery of EP pulses immediately after the R wave detection but within the QRS

complex is the most reasonable. The delivery of EP pulses during the vulnerable period of the atria does not present a serious threat for the patient's life. The hemodynamic effects of atrial flutter and fibrillation, which could be potentially caused by EP pulses during the vulnerable period of the atria, are slim and patients are frequently unaware of them [46]. The time reserve for safe EP pulse delivery after the R wave detection and before the onset of the vulnerable period of the ventricles is approximately 60 ms. This time reserve is long enough for safe EP pulse delivery by plate or needle row electrodes and even avoids the vulnerable period of the atria. The requirement for avoiding the delivery of EP pulses at the moments of potential danger for the patient would be fulfilled excellently as indicated by the ideal $+P_p$ values for all ECG signals (see Table 5). However, when using hexagonal electrodes the synchronization becomes irrelevant due to 200 ms-long EP pulse sequence which extends into the vulnerable period of the atria and ventricles. Therefore, a modification of the existing EP pulse delivery protocol for hexagonal electrodes would be needed for safer application in the immediate vicinity of the heart. The suggested solution is to delay the delivery of EP pulses by approximately one half of the current RR interval, which would result in EP pulses being delivered after the vulnerable period provided that the following heartbeat was normal. However, delayed EP pulses would be delivered exactly within the vulnerable period in case of the appearance of premature heartbeat. The other possibility is to synchronize the switching between the electrodes and partial pulse delivery with ECG.

The statistical comparison of performance of the algorithm between ECG signals recorded during electrochemotherapy and ECG signals from the LTST DB database [30] generally showed no statistically significant differences except for positive predictivity for EP pulse delivery ($+P_p$). This performance measure was significantly better for ECG signals recorded during electrochemotherapy than for ECG signals from LTST DB database thus showing that the algorithm was developed for worser conditions than encountered during clinical application of electroporation. In spite of numerous arrhythmias in some ECG signals from the LTST DB database the algorithm performed excellently [30]. The clinical electrochemotherapy was so far indicated only for patients without clinically significant or severe heart disease, which reflects in ideal value $+P_p = 100\%$ in all ECG signals recorded during electrochemotherapy. However, mostly old patients are included in electrochemotherapy treatment nowadays because of the emerging need for palliative treatment of tumors with electrochemotherapy. With the increasing age of the patients, the probability for encountering pathological ECG is also increasing, therefore, the synchronization of EP pulse delivery with ECG would maximize safety of the patient.

5 Conclusions

Currently used electroporation protocols could interfere with functioning of the heart although no such practical evidence exists till now. Because no systematic study regarding this topic has been done yet, we examined in our study the effects of EP pulses on functioning of the heart. We measured ECG signals during electrochemotherapy and analyzed their characteristics. We found no arrhythmias or other pathological morphological changes due to application of EP pulses. The only demonstrated effect of EP pulses on ECG is a transient RR interval decrease. The facts contributing to a belief that EP pulse delivery during electrochemotherapy cannot affect functioning of the heart are: short EP pulse duration, use of direct current, application mainly on locations relatively distant from the heart (i.e., on extremities), and small inter-electrode distance. On the other hand, there are some open issues regarding electrochemotherapy that need to be considered, for example: EP pulses delivered by plate or needle row electrodes that are not synchronized with ECG could be delivered within the vulnerable period, EP pulses delivered by hexagonal electrodes mainly coincide within the vulnerable period, the threshold levels of the heart for elderly patients are lowered, possible use of electrochemotherapy on patients with clinically significant heart disease, new applications with longer durations and/or higher amplitudes of EP pulses as well as applications involving endoscopic or surgical means to access internal tumors are being developed. Even though no practical evidence for electroporation having an effect on functioning of the heart has been observed so far, we can still maximize safety of the patients by incorporating the algorithm for synchronization of EP pulse delivery with ECG in medical equipment for EP pulse delivery. The usual application of eight EP pulses with duration 100 μs each can benefit from synchronizing delivery of EP pulses with electrocardiogram but this is not the case for hexagonal electrodes or combination of high- and low-voltage EP pulses and pulses with higher amplitudes as used in tumor tissue ablation by irreversible electroporation.

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PAPER II

Early effects of intra-abdominal electrochemotherapy of tumors in liver on functioning of the heart

Barbara Mali[1], Gregor Sersa[2], Damijan Miklavcic[1], Tomaz Jarm[1]

[1] *Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia*

[2] *Institute of Oncology Ljubljana, Ljubljana, Slovenia*

ABSTRACT

Electrochemotherapy (ECT) is an effective local antitumor treatment and reported as a safe method. High voltage electric pulses used in ECT of deep seated tumors could potentially lead to adverse heart-related effects, especially for electroporation (EP) pulses delivered within the vulnerable period of the heart, or if the pulses coincided with certain arrhythmias. We report on the preliminary findings about the influence of novel intra-abdominal ECT of liver tumors on functioning of the heart of three human patients. For this purpose the electrocardiograms (ECG) recorded during the surgical procedure were analyzed. Morphological changes in ECG signals were investigated by analyzing the RR, QR, QT and peak-to-peak QT intervals, and the relations between the changes of these ECG parameters, and the electrical current and energy applied during EP pulse delivery were evaluated. We found occurrence of premature atrial contractions during intravenous administration of bleomycin. On the basis of the 95% statistical prediction interval and on the basis of comparison of average values of these parameters before and during EP pulse delivery, transient decrease of the corrected QT interval was found during EP pulse delivery. We found statistically significant correlation between changes in corrected QT interval and electrical current and energy applied during EP pulse delivery. We suggest that it is possible to affect functioning of the heart by ECT of deep seated tumors but most probably these changes would be transient. No adverse effects due to intra-abdominal ECT were found, which may be due to synchronized EP pulse delivery with ECG, but the probability for complications could increase when using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or in the treatment of tumors in the immediate vicinity of the heart. For this reason, when deep seated tumors are treated, the reliable synchronization of EP pulses with ECG is mandatory. We demonstrated that synchronization protocol currently implemented in medical device for ECT of deep seated tumors does not guarantee delivery

of EP pulses exclusively outside the vulnerable period and in absence of heart arrhythmias. Therefore we suggest improvement of synchronization protocol that will maximize the safety of patients.

Keywords: electrochemotherapy, deep seated tumors, liver, electrocardiogram, synchronization

INTRODUCTION

The combined treatment in which delivery of chemotherapeutic drug is followed by application of high-voltage electric pulses locally to the tumor has been termed electrochemotherapy (ECT). The effect of local delivery of electric pulses causes electroporation, which transiently increases membrane permeability also for molecules, such as bleomycin or cisplatin. ECT has been successfully used in clinics for treatment of cutaneous and subcutaneous tumors irrespective of their histological origin *(Mir et al. 1998; Marty et al. 2006)*. In these studies, a typical ECT protocol involved eight 100-μs-long electroporation (EP) pulses with voltage to distance ratio of up to 1300 V/cm for plate and up to 1000 V/cm for needle electrodes. New ECT applications using endoscopic, percutaneous and surgical means to access internal, deep seated tumors are also being developed where amplitudes of EP pulses reaching up to 3000 V *(Soden et al. 2006; Miklavcic et al. 2010; Mahmood & Gehl 2011; Agerholm-Larsen et al. 2011; Edhemovic et al. 2011)*. Recently, initial treatments of intra-abdominal ECT of tumors in liver have been performed at the Institute of Oncology in Ljubljana.

ECT is reported as an efficient and safe method. No adverse effects have been reported so far. In our previous study, the influence of EP pulses on functioning of the heart for ECT of cutaneous and subcutaneous tumors was investigated and no arrhythmias or other pathological morphological changes due to application of EP pulses were found *(Mali et al. 2008)*. The only demonstrated effect of EP pulses on electrocardiogram (ECG) was a transient RR interval decrease that was however attributed to anxiety and stress of the patient undergoing ECT. However, the situation has changed significantly with treatment of deep seated tumors, in particular when tumors are located close to the heart. High voltage electric pulses used in ECT of deep seated tumors could lead to adverse heart-related effects *(Ball et al. 2010; Thomson 2010; Deodhar et al. 2011)*, especially when delivered within the vulnerable period of the heart (Figure 1) or in case of certain arrhythmias *(Reilly 1998)*. Chemotherapeutic drugs alone can also provoke changes in ECG *(Villani et al. 1994)*.

Externally applied electric pulses delivered outside the vulnerable period have extremely low probability of inducing ventricular fibrillation *(Reilly 1998)*. Therefore the synchronization of EP pulse delivery with absolute refractory period of the heart cycle, which is the safest period for EP pulse delivery, was suggested in order to increase safety of the patient (Figure 1) *(Mali et al. 2008)*. Following our recommendation, the new generation of the clinical device for ECT of deep seated

tumors, Cliniporator Vitae (Igea, Carpi, Italy), includes synchronization of EP pulse delivery with R wave *(Bertacchini et al. 2007; Bertacchini et al. 2010)*. The functioning of this synchronization protocol has not been yet checked up thoroughly whether it includes proper capabilities for ECT treatment of deep seated tumors, like tumors in liver.

The aims of this preliminary study are: (i) to evaluate the influence of administration of chemotherapeutic drug (bleomycin) and EP pulse delivery during intra-abdominal ECT of tumors in liver on functioning of the heart; (ii) to evaluate the relation of changes (if any) of several evaluated ECG parameters (RR, QR, QT and peak-to-peak QT intervals; see Figure 1) with electrical current and energy applied during EP pulse delivery; (iii) to evaluate the functioning of the synchronization protocol currently used in clinical ECT; and (iv) to suggest improved procedure for synchronization and compare its functioning with synchronization protocol currently used in clinical ECT.

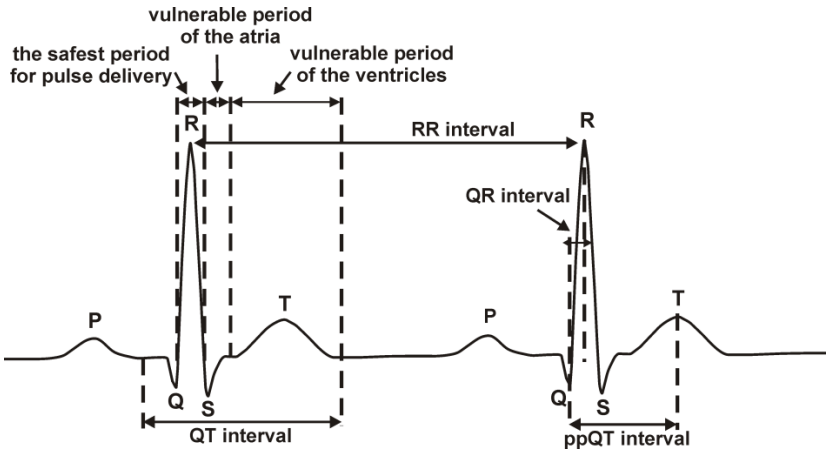

Figure 1: The vulnerable period and evaluated intervals of a typical heartbeat.

MATERIALS AND METHODS

Patients and electrochemotherapy

Three patients (two males and one female, median age 54 years) were treated with intra-abdominal electrochemotherapy (ECT) of metastases of colorectal carcinoma in liver. The treatment was performed under general anesthesia at the Institute of Oncology in Ljubljana and was approved by The National Medical Ethics Committee of the Republic of Slovenia. Bleomycin was administered intravenously (bolus injection) with the dose of 10 mg/m^2 for the first patient and 15 mg/m^2 for the second and third patient. The ECG was recorded during the surgical procedure. None of the patients had a pre-existing cardiac condition. Namely, patients with clinically manifested arrhythmia or with pacemaker are currently excluded from ECT.

Electroporation (EP) pulses were generated by the electric pulse generator Cliniporator Vitae (Igea, Carpi, Italy) that allows the amplitudes of delivered EP pulses up to 3000 V and maximum current of 50 A. Needle electrodes (VG1240) with diameter of 1.2 mm with either 3 or 4 cm long active (conductive) part were used. The delivery of EP pulses was synchronized with ECG via AccuSync (AccuSync 42, AccuSync Medical Research Corporation, USA), a commercially available ECG triggering device. Standard ECG lead II was used in all applications. Trigger signal from AccuSync was further analyzed by the algorithm incorporated in Cliniporator Vitae, which calculated interval between two successive trigger signals. EP pulses were delivered if the value of this interval was within 500 and 3500 ms. ECG and trigger signals (both signals are available as analog outputs on AccuSync) were acquired for further analysis at sampling frequency of 1000 Hz using a Biopac data acquisition and measurement system (Biopac Systems, USA). The recording was started well before the actual procedure and continued after EP pulse delivery, so we have an uninterrupted overview of what was going on during the entire procedure before, during and after ECT.

Altogether 240 EP pulses of 100 µs duration were delivered (128 on the first, 32 on the second, 80 on the third patient). The EP pulses were delivered in trains of eight EP pulses (each pulse synchronized with normal heartbeat) alternately between different pairs of needle electrodes, thus resulting in 30 trains of EP pulses. The minimal interval between two successive trains of EP pulses was 2.5 s (an equivalent of three heartbeats). The number of electrodes, pairs of electrodes among which EP pulses were delivered, distances and voltages applied, were defined according to an individualized treatment planning based on the size of tumor (acquired from CT scan pictures of the treatment area) *(Miklavcic et al. 2010)*. The time course of voltage and current during EP pulse delivery was recorded, which enabled us to calculate values of voltage, current and energy applied during EP pulse delivery.

Analysis of electrocardiogram

The primary analysis of ECG signals recorded during intra-abdominal ECT was made by using QRS detection algorithm based on the analysis of a single lead ECG, developed for synchronization of EP pulse delivery with ECG *(Mali et al. 2005)*. Further analysis of ECG signals was performed to estimate the effect of EP pulse delivery on ECG using program routines as described previously *(Mali et al. 2008)*. As a result, the algorithm extracted RR, QR and QT intervals (Figure 1). Due to more reliable detection of Q and T wave peaks in comparison to the isoelectric level and the end of T wave needed for determination of QT interval, we also calculated the peak-to-peak QT interval (ppQT interval) (Figure 1). The corrected QT and corrected ppQT intervals (QTc and ppQTc interval, respectively)

were calculated as the QT or ppQT interval divided by the square root of the corresponding RR interval.

Four trains of EP pulses (32 EP pulses) were excluded from the analysis due to many small distinctive disturbances included in the ECG during EP pulse delivery that made the detection of heartbeat features unreliable. For the same reason, in six trains of EP pulses only three pulses from a train of eight EP pulses were included in the evaluation. Altogether 178 relatively noiseless heartbeats coinciding with 178 EP pulses were included in the evaluation of changes in ECG.

The updated average of each parameter was calculated from eight values of parameter before EP pulse delivery for each train of EP pulses. Because there were only three heartbeats with no EP pulse delivery between two trains of EP pulses, the updated average in such cases included values of parameter extracted from these three heartbeats and five the newest values included in the previous update average of parameter. The difference between the value of parameter from a heartbeat coinciding with an EP pulse and the corresponding updated average of this parameter was calculated and termed “single-to-mean” difference. For each train of EP pulses we calculated mean of values of this parameter extracted from all heartbeats in this train of EP pulses. Afterwards the difference between this mean and the corresponding updated average of this parameter was also established and termed “mean-to-mean” difference.

Statistical analysis

For evaluation of the effects of EP pulse delivery on ECG, the average values of RR, QR, QTc and ppQTc interval were calculated. We compared average values of these parameters before and during the application of EP pulses using the Wilcoxon Signed Rank test from SigmaPlot 11.0 package. A p value of less than 0.05 was considered as indication of statistically significant difference.

In addition, the prediction interval (PI) was also calculated in order to evaluate the effects of EP pulse delivery on ECG. A PI is an estimate of an interval based on a chosen number of previous observations of certain parameter in which the next future observation of the same parameter is expected to fall with a chosen confidence level. We used a 95% PI, which means that about 95% of the time, the next observation we make will be inside this interval. The PI was calculated from 16 values of evaluated parameters (RR, QR, QTc and ppQTc interval) before EP pulse delivery (Figure 2). One value right before the EP pulse delivery was not included into determination of PI and was used as a control value of this parameter (Figure 2). The PI of parameter was updated for each train of EP pulses. Due to only three heartbeats with no EP pulses between two trains of EP pulses, the updated PI included values of parameter extracted from first two heartbeats of these three and 14 the newest

values from previous PI of parameter (Figure 2). The value of parameter extracted from the third heartbeat was again used as control value of parameter.

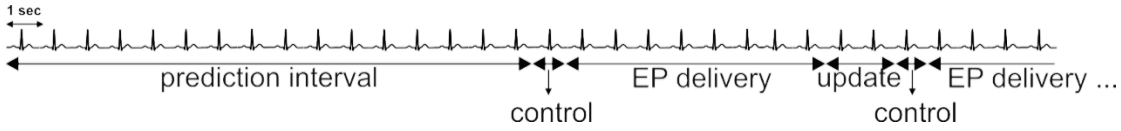

Figure 2: Evaluation of changes due to EP pulse delivery using prediction interval approach

For the degree of association between values of electrical parameters applied during EP pulse delivery and changes of parameters of ECG, linear regression analysis was performed. A *p* value of less than 0.05 was considered as indication of statistically significant correlation. The statistical analysis was performed using SigmaPlot 11.0 package.

Evaluation of synchronization protocols

With the aim to evaluate protocol for synchronization of EP pulses with ECG currently implemented in Cliniporator Vitae device, we examined the ECG and trigger signals recorded during the actual EP pulse delivery. We implemented the algorithm currently used in clinical ECT and evaluated its proper functioning.

We also suggested new decision-making procedure for synchronization of EP pulses with ECG based on previously published algorithm for synchronization of EP pulses with ECG *(Mali et al. 2005)*. Currently the AccuSync trigger is considered valid if it falls within predefined limits of 500 ms and 3500 ms. In our suggested algorithm, the validity of current trigger signal is determined based on an on-line analysis of current RR interval with respect to a regularly updated running average of 8 previous RR intervals (see equation 1). The lower and upper limits for the current RR interval to be considered valid for EP delivery are set as a percentage of the running average of previous RR intervals. The lower limit is stricter than the upper limit. The lower limit is used for the cases when the current RR interval is less than the running average RR. The upper limit is used for the cases where the current RR is larger than running average RR. The lower limit is set to 93% of the running average RR interval (because it is known that premature ventricular contractions can appear as little as 7-10% prematurely according to previous RR interval *(Reilly 1998)*). The upper limit is set less conservatively to 115% of the running average RR interval because extension of RR interval by as much as 15% is (to the best of our knowledge) not associated with potentially harmful arrhythmias. The current RR interval is thus considered valid for EP delivery if:

$$0.93 \cdot \overline{RR_8} \leq RR \leq 1.15 \cdot \overline{RR_8}, \quad (1)$$

where $\overline{RR_8}$ represents a running average of eight previous RR intervals.

The current RR interval is included in calculation of the new, updated running average if fulfils certain requirements. The lower and upper limits for the current RR interval to be included in the updated running average of previous RR intervals are set as a percentage of the running average of previous RR intervals. These lower and upper limits are set to 70% and 130% respectively (equation 2). Only if the current RR interval is within these limits, it will be included in the new running average and the oldest RR interval previously included will be dropped from calculation of the new running average. The current RR interval is thus considered valid for the running average updating (a new $\overline{RR_8}$ will be calculated which includes the current RR interval) if:

$$0.70 \cdot \overline{RR_8} \leq RR \leq 1.30 \cdot \overline{RR_8}\,, \tag{2}$$

where $\overline{RR_8}$ represents a running average of eight previous RR intervals.

Therefore the rules for EP delivery are considerably stricter than the rules for updating of the current running average $\overline{RR_8}$. It means that every RR interval valid for EP delivery is also automatically valid for updating of the running average, but the reverse is not necessarily true.

We implemented our suggested improved procedure for synchronization of EP pulses with ECG. Afterwards, we simulated the delivery of EP pulses for the three patients included in the analysis in order to compare the performance of the currently implemented and our new suggested synchronization algorithm. The purpose was to verify whether our algorithm eliminates (or reduces) errors made (if any) by the present protocol and to see if it introduces any new unwanted effects (such as making the entire ECT procedure longer because of its more conservative nature).

In the simulated procedure the delivery of each treatment session was started at the same moment as it was started during the actual treatment, as evidenced by our ECG records. The simulation also took into account the 2.5 second pause between successive trains of pulses as implemented in the Cliniporator Vitae. We analyzed the results and compared them with the delivery of the pulses that actually took place.

RESULTS

Premature atrial and ventricular contractions were detected during bleomycin administration in ECG signals recorded during intra-abdominal electrochemotherapy (ECT) (Table 1).

No pathological morphological changes caused by electroporation (EP) pulses in patients subjected to intra-abdominal ECT were observed. The comparison of average values of RR, QR, QTc and ppQTc interval before and during EP pulse delivery showed the insignificant change in RR, QR and ppQTc

interval (Table 2). However, the significant decrease was detected in QTc interval duration for each application of EP pulses (Table 2).

In addition, the duration of RR, QR, QTc and ppQTc intervals of one heartbeat before train of EP pulse delivery and all heartbeats during EP pulse delivery were evaluated. For both cases numbers of evaluated parameters that were below the lower and above the upper limit of prediction interval (PI) were determined and the percentage of the values of these parameters falling outside the corresponding PI was calculated. A significant increase in the percentage of QTc values outside the PI was found during EP pulse delivery in comparison to the same value in absence of EP pulse delivery (Table 3). All 86 QTc intervals evaluated during EP pulse delivery that were outside of 95% PI fell out at lower limit of PI, thus showing transient decrease of QTc interval during EP pulse delivery (Table 3).

The linear regression between the single-to-mean difference for RR and QTc interval and current was statistically significant (Table 4). Moreover, statistical significance of linear regression between mean-to-mean and single-to-mean difference for QTc interval and energy was also detected (Table 4).

Currently implemented algorithm for synchronization of EP pulses with ECG signal in Cliniporator Vitae delivered 3 EP pulses at wrong positions (one on a P wave, one just before a P wave, and one right after a T wave) among 240 delivered EP pulses (error rate of 1.25%). The improved algorithm did not deliver EP pulse in any of these three cases. Moreover, it did not introduce any new wrongly delivered EP pulses (error rate of 0%). The algorithm currently implemented in Cliniporator Vitae would definitely deliver pulses on premature atrial and ventricular contractions (Figure 3), whereas the improved algorithm would not deliver EP pulse on any premature beat. The improved algorithm did not induce any significant or clinically relevant extension to the entire procedure. To be precise, in one of the patients the entire procedure was extended by 2.9 seconds. In the other two patients the improved algorithm actually reduced the entire procedure by 1 and 2 seconds, respectively, in comparison to the presently implemented algorithm.

Table 1: The appearance of premature atrial and ventricular contractions (PACs and PVCs respectively) according to the beginning of bleomycin administration (BA) and electroporation pulse delivery (EP) in ECG signals recorded during intra-abdominal electrochemotherapy (ECT)

	before ECT duration: ≈30 min	**during ECT** duration: 20–30 min			**after ECT** duration: ≈5 min
	before BA	during BA	after BA, before EP	during EP	after EP
Patient 1	a sequence of 6 PACs	a sequence of 7 PACs	5 PACs	–	–
Patient 2	–	1 PAC	–	–	–
Patient 3	5 PVCs, 1 PAC	–	–	1 PVC	–

Table 2: The change in average values of heartbeat parameters during EP pulse delivery with respect to values before EP pulse delivery (Wilcoxon Signed Rank test, n = 26).

evaluated parameters [ms]	**median change**	**percentile**		**statistical significance *p***
		25%	**75%**	
RR interval	-4.67	-16.00	5.33	0.112
QR interval	0.00	-1.33	0.67	0.721
QTc interval	-29.2	-36.9	-14.9	<0.001
ppQTc interval	-1.22	-4.42	2.49	0.195

Table 3: Percentage of values of evaluated parameters outside the 95% prediction interval (PI). On left side of the table results for control values of parameters in absence of EP pulse delivery (n = 26) and on right side of the table results for values of parameters during EP pulse delivery (n = 178) are summarized. For both cases numbers of evaluated parameters that were below the lower and above the upper limit of PI (N_{below} and N_{above}, respectively) are given.

evaluated parameters	**in absence of EP**			**during EP**		
	%	N_{below}	N_{above}	%	N_{below}	N_{above}
RR interval	7.69	2	0	29.21	30	22
QR interval	0.00	0	0	7.87	7	7
QTc interval	3.85	1	0	48.31	86	0
ppQTc interval	0.00	0	0	10.67	15	4

Table 4: Statistical significance of linear regression between changes in parameters RR, QR, QTc and ppQTc (dependent variable) and the electric current or energy (independent variable). For evaluation of changes in parameters, for each parameter mean-to-mean difference (n = 26) and single-to-mean difference (n = 178) was considered.

	RR interval		**QR interval**		**QTc interval**		**ppQTc interval**	
	n = 26	n = 178	n = 26	n = 178	n = 26	n = 178	n = 26	n = 178
current	0.053	**<0.001**	0.621	0.860	0.302	**0.001**	0.464	0.219
energy	0.539	0.212	0.944	0.962	**0.029**	**<0.001**	0.453	0.097

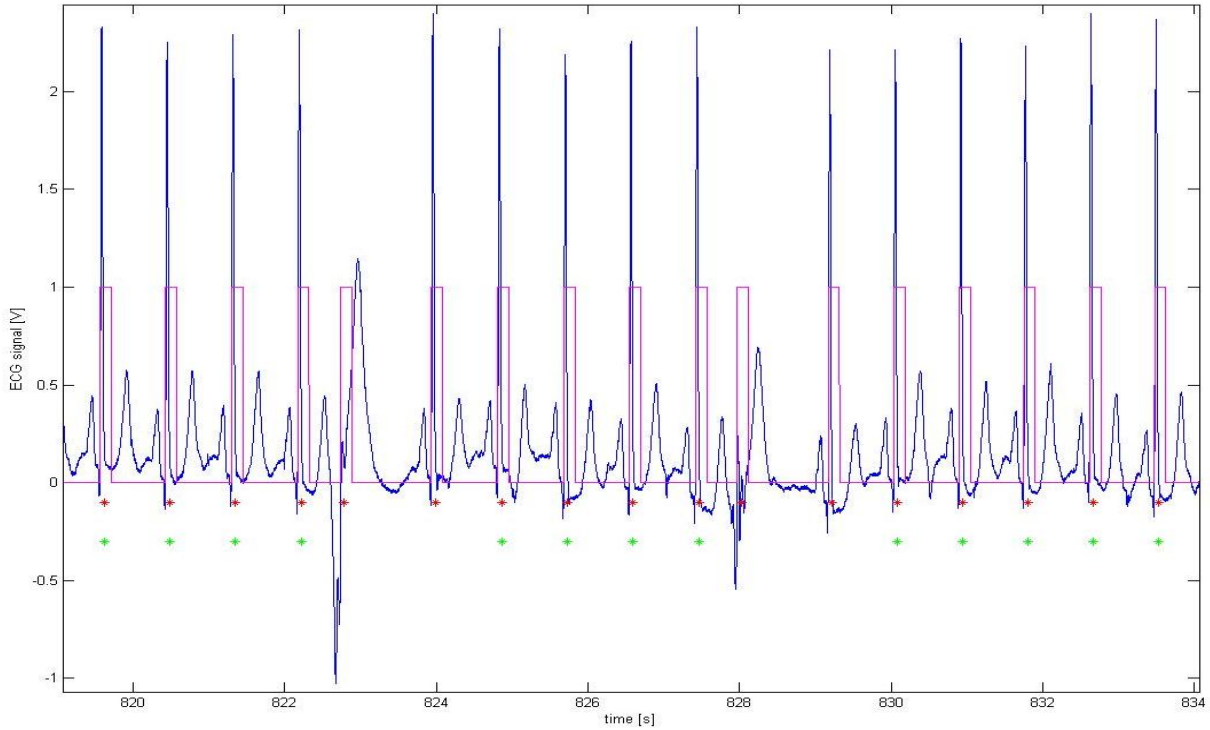

Figure 3: Comparison of delivery of EP pulses for Cliniporator Vitae and for suggested improved algorithm in presence of premature ventricular contractions (5th and 11th heartbeat). Blue line: ECG signal; Magenta line: AccuSync trigger signal; Red asterisks: location of possible EP pulse delivery by the Cliniporator Vitae (trigger pulses considered as valid by the present algorithm); Green asterisks: location of possible EP pulse delivery according to suggested improved algorithm (trigger pulses considered valid by the improved algorithm); Note that the improved algorithm would avoid EP delivery on premature beats and would skip a heartbeat right after the premature contraction (due to a too long RR interval).

DISCUSSION

Our results confirm that intravenous administration of bleomycin can provoke changes in functioning of the heart, expressed as occurrence of premature atrial contractions during bleomycin administration (Table 1). According to the literature, this phenomenon can be attributed to cardiotoxicity of bleomycin *(Villani et al. 1994)*.

QT interval is considered to be an indicator of total duration of ventricular electrical activity. A significant change in QT interval is one of the most important indicators of arrhythmias *(Anderson 2006)*. However, its value depends also on the heart rate (the faster the heart rate, the shorter the QT interval) and has to be adjusted to aid interpretation. For this reason the QTc interval is used in practice. In our study, a transient significant decrease of QTc interval (median value of -29.2 ms) was found, which disappeared immediately after the end of electroporation (EP) pulse delivery (Table 2). Similarly, a significant increase in percentage of values of QTc intervals that fell outside the lower limit of prediction interval (PI) during application of EP pulses in comparison to percentage of values of this parameter that fell outside the PI in absence of EP pulse delivery was detected (increase from 3.85% to 48.31%, Table 3). These significant results could indicate a clinically relevant effect of EP pulse delivery on ECG. However, no significant percentage increase of values of ppQTc interval observed in our study could lead to the opposite assumption – there is clinically irrelevant effect of EP pulse delivery on ECG. Small disturbances sometimes included in ECG signal during EP pulse delivery could aggravate the detection of the end of T wave and thus determination of the QTc interval.

EP pulses were synchronized with the refractory period of the cardiac cycle. Nevertheless, the likelihood of electroporation to influence functioning of the heart depends on the applied electrical current and energy; duration, number and repetition frequency of EP pulses; and electric current pathway *(Reilly 1998)*. Our preliminary results show statistical significance of linear regression between single-to-mean difference for QTc interval, and current or energy (Table 4). In the study by Gomes et al. it was shown that the threshold energy level that stimulates the heart strongly depends on the age of the subject (i.e. old animals have lower threshold levels) *(Gomes et al. 2002)*. Due to high values of energy of applied EP pulses (median value 7.05 J) and decreased threshold energy level for stimulus because of high age of patients (median age 54 years), it is theoretically possible that EP pulse application would lead to heart arrhythmias. This is in agreement with the results of a study by Lavee et al. using irreversible electroporation for epicardial atrial ablation for the treatment of atrial fibrillation that showed that ablation pulses (amplitudes of 1500 to 2000 V, duration 100 µs) caused transient but not permanent arrhythmia or any other rhythm disturbance apart from the

rapid atrial pacing during the pulse sequence application *(Lavee et al. 2007)*. They observed immediate resumption of sinus rhythm following the ablation. However, the results of the work by Al-Khadra et al. showed no arrhythmias in association with electroporation applied directly on the heart *(Al-Khadra et al. 2000)*. According to the heart strength-duration curve a very large current would be required to cause single premature ventricular contraction for very short EP pulse duration (the millisecond range) *(Reilly 1998)*. Since no appearance of premature ventricular contraction due to EP pulses was detected in our preliminary study, it is therefore most improbable that EP pulses alone could create the inhomogeneity (altered states of depolarization-repolarization), which is a prerequisite for the onset of ventricular fibrillation. Therefore it seems possible to affect functioning of the heart by electrochemotherapy (ECT) treatment of deep seated tumors located close to the heart muscle but most probably these changes would only be transient. For this reason, when deep seated tumors are treated, the reliable synchronization of EP pulses with the refractory period of the cardiac cycle in medical equipment for EP pulse delivery should be used, in order to maximize the safety of the patients.

The synchronization protocol currently included in clinical device for ECT of deep seated tumors does not guaranty the delivery of EP pulses exclusively on the R waves (and thus outside the vulnerable period of the heart) and in absence of premature heartbeats (Figure 3), and so potentially dangerous effects for the patient's heart cannot be excluded. Our suggested algorithm for synchronization of EP pulse delivery with ECG is more accurate and safer than the algorithm currently used in clinical ECT. In spite of higher complexity and better performance it also does not increase the duration of ECT procedure. We suggest that synchronization protocol currently implemented in clinical device for ECT should be improved to prevent EP pulse delivery under certain conditions of increased probability for external induction of ventricular fibrillation (such as in case of premature heartbeats).

CONCLUSIONS

In our preliminary study on changes in several ECG parameters (e.g. RR, QR, QTc, peak-to-peak QTc interval) during intra-abdominal electrochemotherapy (ECT) in three patients an occurrence of premature atrial contractions during bleomycin administration and a transient QTc interval decrease during electroporation (EP) pulse delivery was identified. We found statistically significant correlation between changes of QTc interval and electrical current and energy applied during EP pulse delivery. Although no adverse effects due to intra-abdominal ECT were found, most probably due to synchronized electroporation pulse delivery with the electrocardiogram, the probability for complications could increase when using pulses of longer durations or larger number of pulses of increased pulse repetition frequency and/or in treatment of tumors in immediate vicinity of the

heart. The synchronization protocol currently used in clinical ECT of deep seated tumors proved not to have appropriate capabilities for safe and reliable EP pulse delivery; therefore, we suggest a relatively simple change in the synchronization algorithm, which can greatly decrease the probability of inaccurately delivered EP pulses. In our future work, we will evaluate limits of EP pulse characteristics for safe application of ECT in vicinity of the heart. We will also establish potential longer term effects of intra-abdominal ECT of tumors in liver on functioning of the heart.

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PAPER III

Late effects of intra-abdominal electrochemotherapy of tumors in liver on functioning of the heart

Barbara Mali[1], Gregor Sersa[2], Damijan Miklavcic[1], Tomaz Jarm[1]

[1] *Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia*

[2] *Institute of Oncology Ljubljana, Ljubljana, Slovenia*

ABSTRACT

Background. The use of electroporation-based techniques, like electrochemotherapy (ECT), gene electrotransfer and non-thermal irreversible electroporation, is spreading from cutaneous to deep seated tumors. The feasibility of treatment on such tumors has been already confirmed and several clinical studies have been initiated. However, the existence of effect of such treatments on functioning of the heart has not been sufficiently investigated yet, particularly for tumors located close to the heart. The aim of this study was to investigate the existence of effects of intra-abdominal ECT of tumors in liver on functioning of the heart within 24 hours after ECT treatment.

Materials and methods. Four-hours-long night-time ECG signals recorded before and after intra-abdominal ECT on 11 patients were analyzed. Changes in proportion of abnormal heartbeats and heartbeats with ST segment changes, changes in mean values of corrected peak-to-peak QT (ppQTc) interval and changes in median values of various HRV measures (mean NN, SDNN, SDSD, RMSSD, pNN50, SD1, SD2, SD1/SD2, LF, nLF, HF, nHF, LF/HF) due to intra-abdominal ECT were examined based on statistical analysis.

Results. No major late (occurring within 24 hours after ECT treatment) effects (i.e. ventricular tachycardia or fibrillation) on functioning of the heart of patients or post-operative heart arrhythmias due to intra-abdominal ECT were identified. Intra-abdominal ECT has no late effects expressed as change of ST segment. Statistically significant (p <0.001) decrease in proportion of abnormal heartbeats and no significant change in median value of ppQTc interval were identified. Based on HRV analysis, statistically significant decrease in median values of mean NN interval, Poincaré descriptor SD2, low-frequency (LF) and

normalized low-frequency (nLF) component, and increase in normalized high-frequency (nHF) component were observed after intra-abdominal ECT. These changes in HRV measures, however, can most likely be attributed to effects of analgesics and other drugs administered in post-operative care, and post-operative pain. No indication about effects of intra-abdominal ECT itself on functioning of the heart could be found.

Conclusions. Based on the results of this study, we can conclude that the entire procedure of intra-abdominal ECT has late effects on heart rate and long-term HRV. It is not clear whether ECT procedure alone had any late effects. Further study to clarify this question is needed.

Keywords:

electrochemotherapy, intra-abdominal tumors, late effects, electrocardiogram, heart rate variability, metastasis of colorectal carcinoma, liver

INTRODUCTION

Electroporation-based techniques, like electrochemotherapy (ECT), gene electrotransfer and non-thermal irreversible electroporation, are becoming not only an option for treatment of tumors on the body surface but also an option for treatment of internal, deep seated tumors (such as tumors in bones, brain, liver, lung, prostate, kidney, colon and esophagus) using surgical, percutaneous or endoscopic procedures to gain access to the treatment area *(Soden et al. 2006; Miklavcic et al. 2010; Mahmood & Gehl 2011; Agerholm-Larsen et al. 2011; Edhemovic et al. 2011; Ball et al. 2010; Deodhar et al. 2011; Onik et al. 2007; Pech et al. 2011; Tracy et al. 2011; Hojman 2010; Charpentier et al. 2011)*. These advanced treatment procedures thus provide new possibilities for restoring, correcting or modifying physiological functions but, at the same time, because of their novelty, complexity and technical specificity, they may bring along new risks to individual patients. One identified risk is the possibility that application of electroporation (EP) pulses could interfere with functioning of the heart. Namely, in treatment of deep seated tumors, the electrical current delivered during treatment can propagate through a larger volume of tissue surrounding the treated region due to the absence of a protective barrier of the skin, large inter-electrode distances and/or close proximity to the heart (e.g. in liver, lung or esophagus), and relatively large electrical conductivity of internal tissues and organs. There is, therefore, an increased probability of EP pulses affecting cardiac muscle and interfering with functioning of the heart and thus potentially causing early or late heart-related effects.

Currently, study of intra-abdominal ECT of tumors in liver is in progress at Institute of Oncology Ljubljana. These deep seated tumors are located relatively close to the heart and surrounded with highly conductive tissue if compared to tumors located on the skin. Electrodes with 3 or 4 cm long active parts are being used and located with inter-electrode distances up to 3 cm *(Miklavcic et al. 2010; Edhemovic et al. 2011; Pavliha et al. 2012)*. The amplitude of current delivered during intra-abdominal ECT can be up to 50 A. All these facts result in increased probability of interaction of EP pulses with functioning of the heart. Indeed, according to the recently published studies on non-thermal irreversible electroporation, where EP pulses with practically identical characteristics are applied as in intra-abdominal ECT of tumors in liver, different effects on functioning of the heart, expressed as minor or major hemodynamic and cardiologic changes due to unsynchronized irreversible EP pulse delivery can be expected, such as systolic hypertension, supraventricular tachycardia, ventricular tachycardia with pressure drop, ventricular fibrillation, ST segment elevation and changes in T wave *(Ball et al. 2010; Thomson 2010; Deodhar et al. 2011)*. In these studies, major cardiologic changes (ventricular arrhythmias) were terminated with defibrillator, whereas minor effects in general subsided with accomplished EP pulse delivery. Even though delivery of irreversible EP pulses is synchronized with absolute refractory period of the heart cycle, minor cardiac arrhythmias can still be induced *(Deodhar et al. 2011)*. Moreover, authors reported that the ST-T segment elevations that appeared with myocardial ablations using synchronized irreversible EP pulses trended toward the baseline but did not normalize during the 45 min electrocardiogram (ECG) recording after treatment, despite any myocardial injury observed *(Deodhar et al. 2011)*. This fact indicates potential existence of late effects of EP pulse delivery on functioning of the heart, expressed within 24 hours after treatment.

Other factors related to intra-abdominal ECT procedure (e.g. effects of drugs administered to the patient and surgery procedure) also exist that can contribute to potential late effects on functioning of the heart. The influence of these factors can be investigated using heart rate variability (HRV). Namely, HRV is a reflection of many physiological factors modulating the normal rhythm of the heart. These factors include: age, gender, blood pressure, drugs, heart diseases, diabetes, renal failure, surgery procedure, pain, stress, smoking, alcohol, exercise and sleep *(Camm et al. 1996; Berntson et al. 1997; Acharya et al. 2006)*.

Despite the increasing number of ongoing studies involving ECT for treatment of deep seated tumors, no study on potential late effects of ECT procedure on functioning of the heart has been performed to date. In this study, we investigated the existence of effects of intra-abdominal ECT of tumors in liver on functioning of the heart within 24 hours after ECT treatment.

MATERIALS AND METHODS

Patients and electrochemotherapy

Patients from the ongoing phase I/II clinical study with objective to examine feasibility and safety of intra-abdominal electrochemotherapy (ECT) of colorectal metastases in the liver conducted at Institute of Oncology in Ljubljana (EudraCT number: 2008-008290-54; ClinicalTrials.gov: NCT01264952) were included in this study *(Edhemovic et al. 2011)*. Until now, 17 patients (12 males, 5 females) were treated. None of the patients had any serious pre-existing cardiac condition, because intra-abdominal ECT is currently still contraindicated in patients with clinically manifested arrhythmias or with implanted pacemakers.

The treatment was performed under general anesthesia. The induction of anesthesia was achieved with propofol and maintained with sevoflurane and N_20. Analgesics used during surgery were sufentanil and chirocaine. Norcuron was used as relaxant. During post-operative care, patients received opioid analgesics piritramide and sufentanil, and anti-inflammatory analgesic metamizole, local anesthetics chirocaine and ropivacaine, and antiemetic and gastroprokinetic agent metoclopramide. An infusion of vasopressor norepinephrine was also administered in order to maintain blood pressure. At night, patients received another dose of analgesic metamizole and antipsychotic haloperidol.

Intra-abdominal ECT performed under open surgery was predominantly intended for treatment of larger, unresectable or difficult-to-reach tumors in liver. To reach these tumors, needle electrodes of two types were used: electrodes with custom geometry (VG1240, IGEA, Italy) with either 3 or 4 cm long active (conductive) part and individual electrode diameter of 1.2 mm, and electrodes with fixed hexagonal geometry (N-30-HG, IGEA, Italy) with 3 cm long active part, individual electrode diameter of 0.7 mm and distance between outer electrodes of 1.7 cm. The selection between these two types depended on tumor location and size. Four to six electrodes with custom geometry were chosen in case of deeper located or larger tumors, whereas the fixed hexagonal geometry was used for tumors located on the liver surface. When electrodes with custom geometry were used, patient-specific treatment plan was prepared before ECT procedure based on the size and location of tumor in order to define the number and orientation of electrodes, distances between the electrodes, pairs of electrodes among which electroporation (EP) pulses should be delivered, and applied voltages of EP pulses *(Miklavcic et al. 2010; Kos et al. 2010; Edhemovic et al. 2011; Pavliha et al. 2012)*.

ECT procedure was performed in three steps. First step included insertion of needle electrodes into the tumor and tissue surrounding the tumor. During second step, bleomycin was administered intravenously in bolus with the dose of 15 mg/m^2. Third step initiated eight minutes after the bleomycin administration when EP pulses were delivered. EP pulses of 100 µs duration

were delivered in trains of eight pulses alternately between different pairs of electrodes by electric pulse generator Cliniporator Vitae (IGEA, Carpi, Italy).

The delivery of EP pulses was synchronized with ECG via AccuSync 42, an external ECG triggering device (AccuSync, USA). For this purpose, the ECG signal was acquired by AccuSync 42 independently of the regular ECG monitoring performed by the anesthesiologist. The AccuSync 42 detects the R wave of each heartbeat of recorded ECG signal early on the ascending slope of the R wave and provides a trigger signal to Cliniporator Vitae for each individual heartbeat. Cliniporator Vitae delivers EP pulses 50 ms after valid trigger pulse, thus avoiding the so-called vulnerable period of the ventricles (the T-wave). Validation of trigger pulses is performed by a built-in synchronization algorithm.

Three different protocols of EP pulse delivery were used with respect to EP pulse repetition rate, direction of EP pulse delivery and interval between two successive trains of EP pulses. In the first protocol (protocol A), single EP pulse was synchronized with each normal heartbeat and all eight EP pulses between each pair of electrodes were delivered in the same direction. The minimal interval between two successive trains of EP pulses was 2.5 s (an equivalent of three heartbeats). In the second protocol (protocol B), a train of four EP pulses (delivered at 1 kHz repetition rate) was synchronized with each normal heartbeat. Between each pair of electrodes, two trains of four EP pulses were delivered in the opposite directions. The minimal interval between two successive trains of EP pulses was 2.5 s. When EP pulses were delivered using needle electrodes with fixed hexagonal geometry, the third protocol (protocol C) was used where each train of four EP pulses (delivered at 1 kHz repetition rate) was synchronized with each normal heartbeat without pause interval of 2.5 s between the trains. Between each pair of electrodes, two trains of four EP pulses were delivered in the opposite directions.

ECG recording

Ambulatory 9-lead ECG signals (I, II, III, aVR, aVL, aVF, V_1, V_3, V_6) of approximately 24-hours duration were recorded immediately before and after surgery involving intra-abdominal ECT. ECG signals were acquired using a Holter system (SpiderView, ELA Medical, France) and were sampled at 200 Hz. These ECG signals enabled us to investigate potential effects of intra-abdominal ECT on functioning of the heart within 24 hour after the treatment; therefore, these potential effects were called late effects. No ECG recording was possible for the first three patients (patient number 1, 2 and 3). In addition, ECG signals recorded on patients with number 6, 9 and 11 could not be used due to technical reasons. In total, 11 ECG signals were thus appropriate for our study. Main characteristics of these patients and data about treated tumors are presented in Table 1.

ECG analysis

In this study, ECG analysis is based on comparison of conditions before and after intra-abdominal ECT. For this purpose, periods of recorded ECG signals with the most comparable characteristics and minimal activity of the patients are needed. The most comparable and stationary periods of recorded ECG signals were found to be during the night-time when patients are relatively still. The segments of ECG signals between 0:00 and 4:00 a.m. were therefore chosen for analysis in our study. Since surgery involving intra-abdominal ECT was always performed in the morning, two such segments of exactly 24-hour time elapse between them were taken into evaluation for each patient; the first segment was recorded before and the second after intra-abdominal ECT.

The primary analysis of 9-lead 4-hours-long ECG signals recorded before and after ECT during night-time was performed using previously evaluated algorithm described elsewhere *(Mali et al. 2005; Mali et al. 2008)*. For proper functioning of this algorithm, ECG leads with high R wave amplitude and high dynamics within QRS complex in comparison to other parts of ECG signal were required. Therefore, the algorithm was used on ECG signals of leads I, II and V_6. The algorithm provided Q, R and T wave locations and classifications of individual heartbeats (labeled as normal or abnormal, i.e. abnormal in ECG shape or heart rhythm) for each of these three ECG leads. ECG lead on which analysis presented the highest number of normal and the lowest number of abnormal beats was selected as the most appropriate for further analysis. On this ECG lead, the number of ST segments that deviate from normal levels was also identified using multichannel Holter analysis software (SyneScope, ELA Medical, France).

Each 4-hours long ECG segment was further divided into 20-min long subsegments and, finally, one 5-min subsubsegment was extracted from each 20-min subsegment and used for analysis. First 5-min long segment from each 20-min subsegment that included less than 1% of abnormal heartbeats and, at the same time, began at least 10 min after the end of preceding selected 5-min long segment. If no segment could be selected according to these criteria, the criterion for allowed percentage of abnormal beats was increased by 1% until such segment could be determined. This approach enabled extraction of relatively noise-free 5-min long subsegments which are required for reliable analysis of heart rate variability (HRV). Twelve such 5-min-long segments were determined and included in our analysis for each patient for the period before and twelve for the period after ECT treatment.

The selected 5-min-long segments were visually reviewed for correctness of R peak detection, location and heartbeat classification and, if necessary, manually corrected to assure correct identification and classification of every heartbeat. Special software with graphical user interface was developed in Matlab for easier screening and checking for correctness of the located feature points and classification of heartbeats. After screening and editing of R peak data, the

locations of Q and T peaks were also verified but only for heartbeats classified as normal since locations of Q and T peaks of abnormal heartbeats were unreliable and thus inappropriate for the analysis.

Assessment of QT intervals

QT intervals were defined as an interval between the Q peak and the T peak occurrence (peak-to-peak QT intervals, denoted as ppQT) within each individual normal heartbeat. We decided to determine ppQT instead of the standard QT interval, which is defined as time elapsed between the start of Q wave and the end of T wave, as the peaks can be more reliably determined than the start of Q wave and the end of T wave. The corrected ppQT intervals (denoted as ppQTc intervals) were determined for each heartbeat as the ppQT interval divided by the square root of the corresponding RR interval. QT intervals were not determined from ECG signals that contained high-frequency noise on mostly entire ECG signal recorded either before or after ECT, because reliable Q and T peak detection was disabled. Consequently, assessment of ppQT intervals was not performed for patients with such ECG signals.

Assessment of heart rate variability

The HRV analysis is analysis of fluctuations of RR interval time series between adjacent QRS complexes resulting from sinus node depolarization (so-called normal-to-normal (NN) intervals) *(Camm et al. 1996)*. However, the RR interval series obtained from ambulatory ECG recordings are in most cases imperfect, since they also contain abnormal RR intervals. Inclusion of abnormal RR intervals affects substantially the results of statistical time-domain and especially frequency-domain HRV measures *(Camm et al. 1996)*. Therefore, additional preprocessing of RR intervals is needed in order to improve reliability of HRV analysis.

The RR intervals were determined from previously analyzed 5-min-long segments of the ECG signals. Because 5-min long subsegments were chosen in a way that they included 1% or less abnormal heartbeats, also only small proportion of abnormal RR intervals can be expected. Sections including abnormal RR intervals were edited using cubic spline interpolation method, where individual abnormal RR intervals were replaced with the interpolated RR intervals, which proved to be more reliable method that just deleting of such abnormal RR intervals *(Lippman et al. 1994)*. By applying this method, a NN interval sequence appropriate for HRV was obtained. The NN interval sequence is always an irregularly time-sampled sequence. For spectral analysis it should therefore be converted to an equidistantly time-sampled sequence. We applied cubic spline interpolation method at 1000 Hz sample rate and then resample entire NN interval sequence with resample rate of 4 Hz *(Camm et al. 1996)*. This equidistantly sampled NN intervals finally enabled assessment of HRV.

Following the recommendations in the literature for short-term ECG recordings *(Camm et al. 1996)*, several HRV measures in time-domain (mean NN, SDNN, SDSD, RMSSD, pNN50) and frequency-domain (LF, nLF, HF, nHF, LF/HF), and nonlinear measures, i.e. Poincaré descriptors, (SD1, SD2, SD1/SD2) were calculated from NN intervals for each 5-min-long segment included into analysis (for details see Table 2). Linear detrending of NN interval sequences was, in addition, performed before calculation of HRV measures (except for mean NN interval and Poincaré descriptors) to remove the potential low frequency baseline trend component. HRV measures were calculated for all twelve 5-min-long segments of ECG signal recorded either before or after ECT. Afterwards, the median value for each HRV measure was calculated, separately for ECG signals recorded before and after ECT. The comparison of these median values before and after intra-abdominal ECT was used for evaluation of late effects of intra-abdominal ECT on functioning of the heart.

All calculations were performed in Matlab. Frequency-domain HRV measures were calculated with non-parametric FFT-based Welch's periodogram method using Hamming window and with parametric autoregressive-model-based method (AR model of the order 16 with coefficients determined based on Burg method) *(Camm et al. 1996; Acharya et al. 2006)*. Since both approaches provide comparable results, the results of autoregressive-model-based calculation of frequency-domain HRV measures are only reported.

Assessment of electrical parameters of EP pulse delivery

The time course of voltage and current during EP pulse delivery was recorded and stored for further analysis. These records allowed us to determine number of EP pulses delivered on each tumor and to calculate the average values of voltage and current, and energy applied on individual tumor and patient. The average value of voltage and current delivered on each tumor was calculated as median of individual median values of voltage and current delivered within each train of EP pulses. Energy applied on individual tumor was calculated as integral of power, i.e. product of current and voltage, delivered during EP pulse delivery on this tumor. Energy applied on individual patient was determined as the sum of values of energy applied on individual tumors of the patient. The values of these electrical parameters served for determination of the degree of association between them and potential significant changes in ppQTc interval and HRV measures.

Statistical analysis

For evaluation of the effects of intra-abdominal ECT of tumors in liver on functioning of the heart, several statistical comparisons were performed. The proportions of abnormal heartbeats obtained from 4-hour-long ECG signals recorded before and after ECT were compared using Chi-square test. The median values of ppQTc intervals and HRV measures before and after ECT were

compared using paired Wilcoxon Signed Rank Test. In addition, for those evaluated parameters that were found statistically significantly different after intra-abdominal ECT with respect to before ECT procedure, method of linear regression was applied in order to determine the degree of association between different parameters of EP pulse delivery applied on the patients and identified significant changes in evaluated parameters. Different protocols of EP pulse delivery were also compared using one-way ANOVA. The statistical analysis was performed using SigmaPlot 11.0 package. In all tests, a p value of less than 0.05 was considered as indication of statistically significant difference.

Table 1. Main characteristics of the patients and tumors treated with ECT. The two longest perpendicular tumor diameters were determined based on ultrasound images taken during intra-abdominal surgery, just before ECT.

Patient number[#]	Sex	Age	Preexisting cardiac conditions	Tumor type	The two longest perpendicular tumor diameters [cm]
4	M	55	none	metastases of CRC	M1: 1.0 x 1.0 M2: 2.0 x 1.0
5	M	59	none	metastases of CRC	M1: 1.0 x 1.0 M2: 1.5 x 1.2
7	M	69	hypertension	metastases of CRC	M1: 1.7 x 1.0 M2: 1.0 x 1.0
8	F	38	none	metastases of CRC	M1: 1.0 x 1.0 M2: 1.0 x 1.0
10	M	54	none	metastases of CRC	M1: 2.0 x 2.0 M2: 1.0 x 1.0 M3: 0.7 x 0.7
12	F	62	hypertension	metastases of CRC	M1: 3.5 x 3.0 M2: 3.0 x 3.0
13	M	61	hypertension	metastases of CRC	M1: 1.0 x 1.0 M2: 1.0 x 1.0
14	M	63	hypertension	metastasis of CRC	M1: 3.0 x 2.5
15	M	69	hypertension, mild mitral regurgitation	metastases of CRC	M1: 3.5 x 2.5 M2: 1.5 x 1.5
16	F	58	hypertension	metastases of CRC	M1: 2.5 x 2.5 M2: 1.8 x 1.1
17	F	60	none	primary HCC	M1: 1.3 x 1.2

[#] = the original recruitment number of patients included in clinical study (for patients not included see explanation in Methods). CRC = colorectal carcinoma. HCC = hepatocellular carcinoma. M1, M2, M3 = the first, second and third metastasis, respectively.

Table 2. Descriptions of time-domain, nonlinear and frequency-domain HRV measures used in this study.

Parameter	Unit	Description
Time-domain measures		
mean NN	ms	Mean of normal-to-normal (NN) intervals
SDNN	ms	Standard deviation of NN intervals; estimate of overall HRV
SDSD	ms	Standard deviation of differences between adjacent NN intervals; estimate of short-term HRV; describes parasympathetic activity
RMSSD	ms	Square root of the mean of the sum of the squares of differences between adjacent NN intervals; estimate of short-term HRV; describes parasympathetic activity
pNN50	%	Number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals
Nonlinear measures		
SD1	ms	Standard deviation of the Poincaré plot perpendicular to line-of-identity; shortest diameter of the fitted ellipse ; estimate of short-term HRV; describes parasympathetic activity
SD2	ms	Standard deviation of the Poincaré plot along the line-of-identity; longest diameter of the fitted ellipse; estimate of long-term HRV; describes sympathetic activity
SD1/SD2	–	Ratio of SD1 to SD2; describes the ratio of short-term to long-term HRV; describes the relationship between parasympathetic and sympathetic activity
Frequency-domain measures		
LF	ms^2	Power in low frequency range (0.04–0.15 Hz); estimate of long-term HRV; reflects both sympathetic and parasympathetic activity
nLF	n.u.	Normalized power of LF; LF/(LF+HF)x100; estimate of long-term HRV; reflects both sympathetic and parasympathetic activity
HF	ms^2	Power in high frequency range (0.15–0.4 Hz); estimate of short-term HRV; describes parasympathetic (vagal) activity
nHF	n.u.	Normalized power of HF; HF/(LF+HF)x100; estimate of short-term HRV; describes parasympathetic activity
LF/HF	–	Ratio between LF and HF range powers; describes the ratio of long-term to short-term HRV; describes the relationship between sympathetic and parasympathetic activity, i.e. sympathovagal balance

RESULTS

Table 3 presents the details about electroporation (EP) pulse delivery on 11 patients included in the analysis. In most cases leads II and V6 were used for the analysis (Table 4).

No late major adverse effects (i.e. ventricular tachycardia or fibrillation) on functioning of the heart of patients due to intra-abdominal ECT were observed by doctors in post-operative nursing care. The analysis of ECG signals recorded before and after intra-abdominal ECT showed no additional premature heartbeats in ECG signals recorded after ECT even in the cases where premature heartbeats were present in ECG signal before ECT (Table 4). On the contrary, statistically significant decrease (Wilcoxon Signed Rank test, $p = 0.019$) in individual proportions of abnormal heartbeats after intra-abdominal ECT with respect to before ECT procedure was identified (Table 4). No statistically significant difference (paired t-test, $p = 0.687$) between individual proportions of deviated ST segments before and after intra-abdominal ECT was detected (Table 4).

ECG signals recorded on patients with number 7, 8 and 15 contained high-frequency noise with low-amplitude on entire ECG signal recorded either before or after ECT which prevented reliable determination of ppQTc intervals for these patients (Table 4). On remaining eight patients, the comparison of median values of ppQTc interval before and after intra-abdominal ECT indicated no statistically significant difference (Table 5).

All 11 patients were included in HRV analysis. The Wilcoxon Signed Rank Test showed statistically significant decrease in median values of parameters mean NN interval, SD2, LF, and nLF, and increase in median nHF after intra-abdominal ECT (Table 5). Statistically significant negative linear correlation between changes in SD2 and LF parameters, and the number of EP pulses applied during EP pulse delivery was detected ($p = 0.024$ and $p = 0.019$, respectively; Table 6).

When the three different protocols (protocols A, B and C) of EP pulse delivery were compared for each analyzed HRV measure and ppQTc interval, no statistically significant difference was found.

Table 3. Main characteristics of electroporation (EP) pulse delivery for individual patient and tumor.

Patient number	Electrode geometry, length of active part, protocol of EP pulse delivery	No. of electrodes used	No. of delivered EP pulses	Average voltage applied [V]	Average current applied [A]	Energy applied [J]	Total energy applied on patient [J]
4	custom, 3 cm, protocol A	M1: 5 M2: 6	M1: 65 M2: 72	M1: 1695 M2: 1875	M1: 16.69 M2: 19.67	M1: 196.1 M2: 275.1	471.2
5	custom, 3 cm, protocol A	M1: 5 M2: 5	M1: 64 M2: 64	M1: 1052 M2: 1100	M1: 10.58 M2: 11.74	M1: 75.7 M2: 89.6	165.3
7	custom, 3 cm, protocol A	M1: 5 M2: 5	M1: 64 M2: 64	M1: 1924 M2: 1744	M1: 24.40 M2: 17.25	M1: 304.7 M2: 177.7	482.4
8	custom, 3 cm, protocol A	M1: 5 M2: 5	M1: 64 M2: 64	M1: 1778 M2: 1778	M1: 26.82 M2: 24.16	M1: 312.1 M2: 266.4	578.5
10	custom, 3 cm, protocol A	M1: 6 M2: 5 M3: 4	M1: 110 M2: 64 M3: 53	M1: 2810 M2: 2131 M3: 2024	M1: 29.72 M2: 21.89 M3: 31.21	M1: 867.2 M2: 314.4 M3: 386.3	1567.9
12	custom, 3 cm, protocol A	M1: 6 M2: 6	M1: 104 M2: 111	M1: 2533 M2: –	M1: 31.50 M2: –	M1: 802.6 M2: –	1605.2[#]
13	hexagonal, 3 cm, protocol C	M1: 7 M2: 7	M1: 96 M2: 288	M1: 718 M2: 718	M1: 4.59 M2: 3.77	M1: 32.0 M2: 81.5	113.5
14	custom, 3 cm, protocol B	M1: 5	M1: 75	M1: 2421	M1: 46.13	M1: 5976.0	5976.0
15	hexagonal, 3 cm, protocol C	M1: 7 M2: 7	M1: 672 M2: 96	M1: 713 M2: 713	M1: 6.67 M2: 6.30	M1: 321.8 M2: 47.4	369.2
16	hexagonal, 3 cm, protocol C	M1: 7 M2: 7	M1: 288 M2: 384	M1: 713 M2: 713	M1: 9.02 M2: 8.24	M1: 184.4 M2: 222.0	406.4
17	custom, 3 cm, protocol B	M1: 5	M1: 64	M1: 1883	M1: 27.70	M1: 318.1	318.1

M1, M2, M3 = the first, second and third metastasis, respectively. – = no data. [$] = estimated value calculated as double energy applied on tumor M1 due to almost identical parameters of EP pulse delivery used on both tumors.

Table 4. Analyzed ECG leads and heartbeat intervals (normal-to-normal (NN) intervals, corrected peak-to-peak QT (ppQTc) interval), and number of heartbeats classified as normal (N) or abnormal (A) and with ST segment deviation (ST) in 4-hours long night-time ECG signal recorded before and after intra-abdominal electrochemotherapy (ECT).

Patient number	ECG lead used for analysis before/after ECT	Analyzed heartbeat intervals	No. of beats before ECT			No. of beats after ECT		
			N	A	ST	N	A	ST
4	II / II	NN, ppQTc	14317	12	0	14216	17	0
5	II / II	NN, ppQTc	16873	20	0	18820	15	2321
7	V_6 / I	NN	14785	70	0	15202	23	763
8	II / V_6	NN	18321	18	6915	26047	9	0
10	II / II	NN, ppQTc	15722	3	15692	18112	1	14001
12	II / II	NN, ppQTc	17461	25	696	26450	39	25188
13	V_6 / V_6	NN, ppQTc	13857	135	10111	16620	0	7078
14	II / II	NN, ppQTc	13864	6	0	19405	8	3209
15	II / II	NN	13975	309	0	18271	8	0
16	V_6 / V_6	NN, ppQTc	18825	42	0	17764	6	4395
17	II / II	NN, ppQTc	17125	8	9279	20635	6	8715
	Summary (%)		175125 (99.63)	648* (0.37)	42693# (24.29)	211542 (99.94)	132* (0.06)	65670# (31.02)

* = significance of Wilcoxon Signed Rank test p = 0.019; # = significance of paired t-test p = 0.687

Table 5. The list of median changes in mean peak-to peak corrected QT interval (ppQTc interval) and time-domain, nonlinear and frequency domain HRV measures after intra-abdominal ECT, and their statistical significance.

Evaluated parameters	Median change	Percentile		Statistical significance of change (*p*)*
		25%	75%	
mean ppQTc [ms]	-9.38	-18.07	1.29	0.313
mean NN [ms]	-171.13	-253.26	-39.40	**0.010**
SDNN [ms]	-10.50	-17.69	-4.07	0.102
SDSD [ms]	-4.20	-9.92	0.143	0.148
RMSSD [ms]	-4.19	-9.90	0.147	0.148
pNN50 [%]	-0.17	-1.31	0.12	0.426
SD1 [ms]	-2.97	-7.01	0.101	0.148
SD2 [ms]	-17.52	-26.08	-4.16	**0.042**
SD1/SD2	0.01	-0.09	0.11	0.638
LF [ms^2]	-94.88	-270.88	-30.93	**0.032**
HF [ms^2]	-14.87	-47.79	28.52	0.465
nLF [n.u.]	-12.30	-15.83	-5.57	**0.042**
nHF [n.u.]	12.30	5.57	15.83	**0.042**
LF/HF	-1.04	-2.78	-0.65	0.320

*Wilcoxon Signed Rank Test

Table 6. Statistical significance of linear regression between changes (**Δ**) in parameters that were statistically significantly different after intra-abdominal ECT with respect to before ECT procedure, i.e. mean NN, SD2, LF, nLF and nHF (see Table 5), (dependent variable) and different parameters of EP pulse delivery applied on the patients (number of EP pulses applied during EP pulse delivery, the largest average voltage (U) and current (I) applied on tumors on individual patient; total energy per patient) (independent variable).

Compared parameters	No. of delivered EP pulses	The largest average U applied on tumors	The largest average I applied on tumors	Total energy applied on patient
Δ mean NN	0.813	0.354	0.166	0.166
Δ SD2	**0.024**	0.181	0.343	0.879
Δ SD1/SD2	0.439	0.301	0.124	0.223
Δ LF	**0.019**	0.277	0.475	0.984
Δ nLF	0.186	0.572	0.224	0.063
Δ nHF	0.186	0.572	0.224	0.063

DISCUSSION

In this study, no major late effects (i.e. ventricular tachycardia or fibrillation) on functioning of the heart of patients were found due to intra-abdominal electrochemotherapy (ECT).

According to the literature, surgery and anesthesia produce a stress response characterized by increased sympathetic and hormonal activity which may predispose the patient to arrhythmias *(Furuya et al. 1993)*. Post-operative arrhythmias are quite common and affect about 7% of patients that underwent major non-cardiothoracic surgery *(Walsh et al. 2006; Walsh et al. 2007)*. Post-operative arrhythmias occur in older patients, most often in the first 4 days after surgery and are frequently associated with other underlying complications *(Walsh et al. 2006; Walsh et al. 2007)*. In addition, electroporation (EP) pulses alone delivered on liver tissue that is located near the heart can induce abnormal heartbeats, in spite of the fact that EP pulses were synchronized with the refractory period of the cardiac cycle *(Lavee et al. 2007; Ball et al. 2010; Deodhar et al. 2011)*. Moreover, the cardiotoxic effects of bleomycin, the chemotherapeutic drug used in intra-abdominal ECT, can also provoke changes in ECG often manifested as an appearance of, or an increase in the incidence of premature atrial contractions, as appearance of supraventricular tachycardia, bradycardia or as conduction abnormalities *(Villani et al. 1994)*. However, the abnormal heartbeats caused by bleomycin may occur transiently during and shortly after drug administration, and would thus disappear until night-time when we evaluate late effects. In this study, no increase in number of abnormal heartbeats after intra-abdominal ECT was identified. On the contrary, the number of abnormal heartbeats decreased significantly after intra-abdominal ECT (Table 4), which suggests that intra-abdominal ECT has no late side effects expressed as appearance of new-onset post-operative arrhythmias.

EP pulses can also harm myocardium and consequently cause ST segment changes. ST segment changes are a clearly documented occurrence also after cardioversion and defibrillation *(Chun et al. 1981; Jones & Jones 1984; Eysmann et al. 1986; Van Gelder et al. 1991; Reddy et al. 1997; Kok et al. 2000; Wang et al. 2003)*, and were recognized to be transient (in range between few seconds and 24 hours) and not associated with myocardial injury. Our results show no statistically significant change in proportion of heartbeats with ST segment deviation in ECG signals recorded after intra-abdominal ECT with respect to ECG signals recorded before ECT procedure (Table 4). We can conclude that intra-abdominal ECT has no late effects expressed as change of ST segment and causes no myocardial injury.

Heart rate variability (HRV) analysis is a method for study of physiological mechanisms responsible for the control of heart rate, in which the autonomic nervous system appears to play a primary role *(Camm et al. 1996; Berntson et al. 1997; Acharya et al. 2006)*. HRV measures strongly

depend on conditions under which ECG signals are recorded and are affected by many physiological factors modulating the normal rhythm of the heart (such as age and gender of the patient, blood pressure, drugs, heart diseases, diabetes, renal failure, surgery procedure, pain, stress, smoking, alcohol, exercise and sleep) *(Acharya et al. 2006)*. However, when HRV measures are derived from stable ECG signal recorded under controlled, resting conditions, it is suggested that HRV is a reliable measurement that can provide information of the degree of autonomic modulation of the heart *(Sandercock et al. 2005)*. In our study, ECG signals recorded in the same time window during night when patients were lying relatively still (from 0:00 to 4:00 a.m.) were chosen for evaluation before and after intra-abdominal ECT in order to exclude the influence of circadian variation on HRV. Because HRV measures were compared for each patient before and after ECT, age and gender dependence of HRV was eliminated. None of the patients had clinically significant preexisting cardiac condition (Table 1). The study population was relatively homogeneous, i.e. patients with tumors in liver, pretreated with surgery and chemotherapy. As far as the ECT procedure is concerned, the patients were subjected to practically the same intervention: the same type of anesthesia and analgesia used during surgery; comparable surgery section performed; comparable duration of the procedure; ECT performed with intravenously administered bleomycin; similar protocols of EP pulse delivery used; and comparable pre- and post-operative care of the patients. Despite comparable ECT procedure on patients and many comparable conditions (age, gender, night-time period, homogeneous study population) during ECG recordings before and after intra-abdominal ECT were assured, HRV analysis showed statistically significant change in median values of several HRV measures after intra-abdominal ECT (Table 5). This result indicates that intra-abdominal ECT procedure as a whole caused some significant changes in HRV characteristics. These late effects could be attributed either to effect of anesthesia, post-operative drugs, post-operative pain, or ECT.

Induction and maintenance of general anesthesia with sevoflurane and N_2O are known to cause a decrease in both the LF and HF components of the HRV power spectra *(Latson et al. 1992; Donchin et al. 1985; Nakatsuka et al. 2002; Kanaya et al. 2003; Tanaka & Nishikawa 2005; Mazzeo et al. 2011)*. The degree of change tends to depend on the concentration of sevoflurane being used *(Nakatsuka et al. 2002)*. The recovery period of HRV after general anesthesia with sevoflurane was reported to be short (from 30 minutes to 180 min) *(Donchin et al. 1985; Tanaka & Nishikawa 2005; Fujisawa et al. 2009)*. The time elapsed between end of surgery and ECG recording after surgery was about 10 hours; therefore, according to the literature, no significant effects of anesthesia on HRV are expected.

According to the literature, post-operative analgesics, especially opioids, depressed the LF component more than the HF component, and decreased LF/HF ratio, suggesting parasympathetic dominance *(Michaloudis et al. 1998; Kanaya et al. 2003)*. Therefore, the observed significant

decrease of LF and nLF component (estimate of long-term HRV) after intra-abdominal ECT in our study could be explained by the fact that the patients received several analgesics during post-operative care (i.e. piritramide, sufentanil, metamizole). Furthermore, our finding regarding statistically significant decrease of nonlinear HRV measure SD2 after intra-abdominal ECT is consistent with decrease of LF and nLF component, because SD2 parameter estimates long-term HRV as well and correlates with LF domain in HRV *(Camm et al. 1996)*.

Surgical pain and post-operative pain is known to trigger autonomic nervous and endocrine responses, which can be manifested as accelerated heart rates, increased blood pressure, reduced blood flow in local tissue, changes in immune response, hyperglycemia, lipolysis, negative nitrogen balance, increased metabolic activity, and increased oxygen consumption *(Seitsonen et al. 2005; Terkelsen et al. 2005)*. Consequently, shortened NN intervals and elevation of the LF component were detected in previous studies, but SDNN, HF, and total power of HRV were not found to be affected by pain *(Seitsonen et al. 2005; Terkelsen et al. 2005; Chang et al. 2012)*. In this study, the analgesics in post-operative care probably did not eliminate the pain perception entirely, which could reflect in statistically significant decrease in mean NN interval after intra-abdominal ECT. In addition, heart rate was probably increased also due to infusion of vasopressor norepinephrine administered to patients in post-operative care because norepinephrine is cardiac stimulator and increases heart rate and blood pressure *(Guyton & Hall 2006)*. Due to acceleration in heart rate, increased short-term HRV and consequently increase of LF component were also expected, which, however, was not detected in our study. This disagreement could probably be explained with the effect of post-operative analgesics that decreased LF component but not completely eliminated the pain perception.

ECT could have effects on HRV due to bleomycin administration and EP pulse delivery. About 70% of the administered intravenous dose of bleomycin is eliminated from the body within 24 hours *(Alberts et al. 1978; Hall et al. 1982)*; therefore, some effects on HRV could still be detected in ECG signals recorded during night-time after intra-abdominal ECT, which is about 11 hours after bleomycin administration during ECT procedure. In the study by Nuver et al, some long-lasting effects of chemotherapy with bleomycin were reported, including a decrease in mean systolic and diastolic blood pressure, and an increase in heart rate, even up to 10 weeks after the last of four courses of chemotherapy *(Nuver et al. 2005)*. Similarly as surgery section or any other pain stimulation are reported to increase the heart rate, delivery of EP pulses could also increase the heart rate but these effects are reported to be only transient and were thus not expected to be present during the night-time after intra-abdominal ECT *(Yasuda et al. 1991; Terkelsen et al. 2005)*. Our results could indicate the potential influence of EP pulse delivery on functioning of the heart due to identified statistically

significant negative correlation between changes in SD2 and LF, and number of delivered EP pulses on a patient (Table 6).

Based on results of our study, we can conclude that no major late (occurring within 24 hours after ECT treatment) effects (i.e. ventricular tachycardia or fibrillation) on functioning of the heart of patients due to intra-abdominal ECT were identified. However, some statistically significant differences in HRV measures after intra-abdominal ECT were detected which could most likely be attributed to effects of post-operative drugs and pain. In order to distinguish the effects of intra-abdominal ECT itself (if there are any) from those of post-operative drugs and pain, the ECG recordings before and after a similar surgical procedure but without ECT would be required, preferably obtained from the same patients on which the ECT has been performed during a previous surgical procedure. However, cases where a surgical procedure involving intra-abdominal ECT is followed by a similar surgical procedure without ECT are very rare and were not available for this study. In addition, for reliable investigation about influence of different parameters of EP pulse delivery (such as number of delivered EP pulses, voltage, current, energy, and different protocols of EP pulse delivery), a detailed data about distance between location of EP pulse delivery and the heart would also be needed.

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PAPER IV

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Electrochemotherapy: A New Technological Approach in Treatment of Metastases in the Liver

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Electrochemotherapy is now in development for treatment of deep-seated tumors, like in bones and internal organs, such as liver. The technology is available with a newly developed electric pulse generator and long needle electrodes; however the procedures for the treatment are not standardized yet. In order to describe the treatment procedure, including treatment planning, within the ongoing clinical study, a case of successful treatment of a solitary metastasis in the liver of colorectal cancer is presented. The procedure was performed intraoperatively by inserting long needle electrodes, two in the center of the tumor and four around the tumor into the normal tissue. The insertion of electrodes proved to be feasible and was done according to the treatment plan, prepared by numerical modeling. After intravenous bolus injection of bleomycin the tumor was exposed to electric pulses. The delivery of the electric pulses did not interfere with functioning of the heart, since the pulses were synchronized with electrocardiogram in order to be delivered outside the vulnerable period of the ventricles. Also the post treatment period was uneventful without side effects. Re-operation of the treated metastasis demonstrated feasibility of the reoperation, without secondary effects of electrochemotherapy on normal tissue. Good antitumor effectiveness with complete tumor destruction was confirmed with histological analysis. The patient is disease-free 16 months after the procedure. In conclusion, treatment procedure for electrochemotherapy proved to be a feasible technological approach for treatment of liver metastasis. Due to the absence of the side effects and the first complete destruction of the treated tumor, treatment procedure for electrochemotherapy seems to be a safe method for treatment of liver metastases with good treatment effectiveness even in difficult-to-reach locations.

Key words: Electrochemotherapy; Liver metastases; Colorectal cancer.

I. Edhemovic, M.D., M.Sc.[1]
E. M. Gadzijev, M.D., Ph.D.[1]
E. Brecelj, M.D., Ph.D.[1]
D. Miklavcic, Ph.D.[2]
B. Kos, B.Sc.[2]
A. Zupanic, Ph.D.[2]
B. Mali, B.Sc.[2]
T. Jarm, Ph.D.[2]
D. Pavliha, B.Sc.[2]
M. Marcan, B.Sc.[2]
G. Gasljevic, M.D.[1]
V. Gorjup, M.D., Ph.D.[3]
M. Music, M.D., Ph.D.[1]
T. Pecnik Vavpotic, B.Sc.[1]
M. Cemazar, Ph.D.[1]
M. Snoj, M.D., Ph.D.[1]
G. Sersa, Ph.D.[1]*

[1]Institute of Oncology Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia
[2]University of Ljubljana, Faculty of Electrical Engineering, Trzaska 25, SI-1000 Ljubljana, Slovenia
[3]University Medical Center Ljubljana, Zaloska 2, SI-1000 Ljubljana, Slovenia

Introduction

Electrochemotherapy is a local treatment that uses electroporation of the tumors to increase uptake of cytotoxic drugs, such as bleomycin or cisplatin (1). In the case of bleomycin, up to a 1000-fold increase in cytotoxicity was observed (1-3). Currently electrochemotherapy is used in treatment of cutaneous and subcutaneous tumors of different histological types with response rate of 80% and long lasting complete responses of 70% (4, 5). The treatment has been predominantly used with palliative intent for melanoma metastases and other cutaneous tumors, whereas the colorectal liver metastases (CRLM) have not been treated yet. In a preclinical *in vitro* study on CMT-93 colorectal carcinoma cells it was demonstrated that exposure of cells to electric field potentiates cytotoxicity of bleomycin 500-fold (6).

Abbreviations: CRLM: Colorectal Liver Metastases; IVC: Inferior Vena Cava; Sg: Segment; MHV: Middle Hepatic Vein; LHV: Left Hepatic Vein; sRHV: Superior Hepatic Vein; US: Ultrasound; CEA: Carcinoembryonic Antigen; H&E: Haematoxilin and Eosin; ECG: Electrocardiogram.

*Corresponding author:
Prof. Gregor Sersa
E-mail: gsersa@onko-i.si

Further development of electrochemotherapy is oriented into treatment of bigger as well as deep-seated tumors; in internal organs or at least 1 cm below the skin. The first reported case was treatment of deep-seated melanoma metastasis in the thigh (7), in which the treatment feasibility was demonstrated, but also a sensitivity study was performed which demonstrated that positioning of electrodes with respect to the initial treatment plan is important for the success of the treatment (8). The treatment of deep-seated tumors is now possible with the new electroporation device and the newly developed long-needle electrodes. In contrast to cutaneous tumor lesions, the abdominal tumors (such as metastases in the liver) are located in electrically highly conductive medium and the treatment region can be in close proximity of the heart. Therefore, the delivery of electroporation pulses should be synchronized with the refractory period of the cardiac cycle to minimize the probability of interaction of electric pulses with the heart function (9-11).

CRLM located in between inferior vena cava (IVC) and the main hepatic veins represent a challenge for a liver surgeon. Although the paracaval region and caudate process in the angle of the main hepatic veins inflow into IVC can be approached with segmental resection of segment 1 (Sg 1) it is often impossible to achieve a sufficient safety margin due to veins proximity. Available alternatives depend on the hepatic veins involved: if both, the middle (MHV) and left hepatic veins (LHV) are involved or the common trunk is involved, extended left hemihepatecomy together with Sg 1 (caudate lobe) is possible. When the superior right hepatic vein (sRHV) and MHV are involved and the LHV is unaffected, the right trisectionectomy may be performed (12, 13). It has become accepted as surgical standard that as much liver tissue as possible is preserved in treatment of CRLM (14). Another possibility; if both sRHV and common trunk are involved, in rare cases, when there are strong inferior and/or middle right hepatic veins present (15), the liver resection which includes left hemihepatecomy and Sg 8 and Sg 7 with preservation of Sg 5 and Sg 4 is possible. Using ablative method like RFA in this region has limitations because of the heat sink from cooling effect of hepatic veins (16-18).

Here we describe for the first time the treatment procedure of electrochemotherapy of CRLM, in a case of a patient with liver metastasis in between IVC and the origin of the main hepatic veins. We demonstrate the feasibility of the procedure and describe the procedure steps needed for electrochemotherapy, safety of the treatment and the treatment effectiveness.

Material and Methods

Clinical Study

In the on-going Phase I/II study (EudraCT number 2008-008290-54; ClinicalTrials.gov (NCT01264952)) so far 8 patients were recruited: 5 patients with CRLM in both hemilivers planned for two-step surgical procedure and 3 patients with solitary metastasis on the MHV inflow to the IVC. The study was approved by Institutional Medical Board and Ethical Committee of the Republic of Slovenia.

Patient

The patient was a 55 years old lady who had been operated for sigmoid colon carcinoma (July 2007), stage pT3, N2 (6 positive out of 19 removed lymph nodes), M0. A R0 resection of the colon was performed, followed by 8 cycles of chemotherapy with Capecitabine. After 24 months a hypoechoic lesion close to IVC was found on ultrasound (US), measuring 24 × 21 mm. The lesion was confirmed to be a metastasis by the PET/CT and MRI scans. It was located in between the IVC and the main hepatic veins. The metastasis increased in a month to 35 × 20 mm. Due to its direct contact with IVC, MHV and sRHV, the metastasis was considered as non-resectable. Chemotherapy (capecitabine and oxalipanine (XELOX) + bevacizumab) was introduced. After three cycles of chemotherapy, in the mid of November 2009, MRI using liver specific contrast Gd-EOB-DTPA showed partial downsizing of the lesion to 34 × 15 mm. It was still close to IVC and main hepatic veins; however there was a possibility that it could have become resectable. RFA was not considered as an option due to the proximity of the IVC and main hepatic veins and their cooling effect. The patient was offered an exploration and electrochemotherapy if the metastasis would still be found non-resectable during the surgery in the end of December 2009. The patient agreed and signed informed consent for electrochemotherapy.

Radiology of the Metastasis

Before electrochemotherapy, the liver metastasis was evaluated by contrast-enhanced Gd-EOB-DTPA MRI of liver. Pre contrast enhanced images included in- and opposed phase transversal images, T1W transversal images and T2W transversal and coronal images. Contrast enhanced images were obtained in late arterial (25 s), portal-venous (70 s) and late phase (5 and 20 min) in transversal and coronal planes. Post treatment the effect was evaluated by multislice contrast enhanced computed tomography (CT) in late portal phase (70 s), 5 mm slice reconstructions were made, in axial and coronary planes.

Treatment Planning

Prior to surgery and electrochemotherapy, numerical treatment planning was performed using a method established and reported earlier (7, 8, 19). Briefly, a 3-D model geometry was built based on segmented MRI images of the patient as described previously (20). The images were segmented into

three tissues: liver, tumor and blood vessels, the IVC and the main hepatic veins. Next, several different electrode configurations were designed in consultation with the surgeon based on the limited number of possible access routes. Using an optimization algorithm coupled with a finite-element model of electroporation, the minimum required voltage for each electrode pair in each electrode configuration was computed to guarantee adequate electric field distribution in the tumor, as this is the major indication for successful electroporation (21). In contrast to previous work (7), a gradient optimization algorithm was used to optimize the voltages between each electrode pair, while positions of electrodes were determined by using the so-called forward modeling approach. Finally, the optimal design using 6 electrodes was used, as it provided the most robust treatment, and the surgeon was able to execute the plan.

Electrochemotherapy

On the exposed liver the needle electrodes (1.2 mm in diameter with 4 cm non-isolated tip length) were inserted into the metastasis under US guidance. The positioning followed the provided treatment plan. The electrodes were connected to the electric pulse generator (Cliniporator *VITAE*, IGEA SpA, Carpi, Italy). Thereafter, the patient was given 15,000 U/m^2 of bleomycin (27.45 mg) intravenously in bolus. Eight electric pulses of 100 µs duration were delivered between pairs of electrodes 8 min after the bleomycin injection, when the maximal pharmacological peak of bleomycin in the metastases was expected (22). The amplitudes and protocol of electric pulses delivery according to the treatment plan for this particular metastasis is given in Table I.

ECG Synchronization

Delivery of electroporation pulses was synchronized with electrocardiogram (ECG); one pulse per heart-beat was delivered. Namely, Cliniporator *VITAE* provides an option for synchronization of electroporation pulse delivery with ECG. The ECG triggering device AccuSync 42 (AccuSync Medical Research Corp., Milford, CT, USA) was used. The AccuSync is a 3-lead electrocardiograph which detects the R-wave from one of the preselected standard leads early on the ascending slope of the R-wave. ECG lead II was selected due to prominence of the R wave. This ECG signal was acquired independently of the regular ECG monitoring performed by the anesthesiologist. The Cliniporator *VITAE* is programmed to deliver a single electroporation pulse 50 ms after receiving a valid trigger from the AccuSync (provided that the latest R-R interval was within the 0.5-3.5 s range) thus avoiding the so-called vulnerable period of the ventricles, the T wave. Both the ECG signal used for synchronization and the trigger signal were recorded and stored for post processing and further analysis.

Surgery

1st Operation – Electrochemotherapy: One month after the third cycle of chemotherapy (XELOX + bevacizumab), the patient was operated. Despite the downsizing which was visible on MRI images, intraoperative US assessment still showed direct contact of the metastasis with the IVC, and main hepatic veins at their origin, so the lesion was considered non-resectable. Due to the reasons explained earlier, RFA was not an option, so electrochemotherapy remained the only possibility. After the mobilization of the left liver, the area between IVC and the origin of the main hepatic veins was exposed. The electrodes were placed and electrochemotherapy was performed, as described. No single adverse effect was noted and blood loss was minimal. Postoperative course was uneventful and the patient was discharged from hospital on the day 10. After this operation patient did not receive any systemic treatment.

2nd Operation – Excision of the Metastasis: Due to changes visible on CT scan (homogenously hypo dense lesion, without

Table I

Summary of planned voltages, number of pulses and predicted currents based on numerical model as well as the actually delivered voltages, number of pulses and measured currents.

Electrode pair	Voltage according to plan [V]	No. of pulses according to plan	Predicted current [A]	Delivered voltage [V]	Delivered No. of pulses	Measured current [A]
1-5	2100	8	31	1300	20	32.3
1-6	2100	8	26	2100	8	45.2
2-5	2100	8	26	1700	21	44.7
2-6	2100	8	25	2100	8	48.3
3-5	2100	8	25	2100	8	48.9
3-6	2100	8	29	1900	8	48.8
4-5	2100	8	28	2100	8	47.5
4-6	2100	8	33	1700	16	41.2
5-6	1700	8	40	1700	8	48.9
Total		72			105	

changes in size) the second operation was performed three months after the first one. Furthermore, intraoperative US scan showed that the metastasis was hypo-echogenic, which was interpreted as probable necrotic changes. The metastasis was found no longer firmly fixed to the surrounding structures, so it was decided to excise it. Postoperatively, a moderate subcutaneous wound infection occurred, which was treated with partial wound dehiscence. No other adverse effects were noted.

Histology

The excised tissue was fixed in 10% buffered formalin. After the macroscopic examination, the specimen was sectioned and entirely taken for microscopic examination. Tissue was embedded in paraffin; 3 μm thick sections were cut and stained using haematoxilin and eosin (H&E). Immunohistochemical studies were performed by peroxidase avidin-biotin method using the formalin fixed and paraffin embedded material. The following primary antibodies were used: against carcinoembyrionic antigen (CEA) (DAKO, Denmark; polyclonal; dilution1:8000) and CK20 (DAKO, Denmark; monoclonal; dilution 1:20) for staining tumor tissue and Hepat (DAKO, Denmark; polyclonal; dilution 1:20) for staining liver tissue.

Results

Location of the Liver Metastasis

The metastasis treated with electrochemotherapy was confirmed by MRI using liver specific contrast Gd-EOB-DTPA before the operation (Figure 1). Twenty minutes after contrast application, a metastasis (34 mm × 15 mm) located in the paracaval region and caudate process, in contact with IVC and main hepatic veins was identified. No other lesions were seen in the liver.

Treatment Plan

Several treatment plans were prepared based on the MRI of the patient. The treatment plans were evaluated based on the quality of the predicted electric field distribution (for details see ref. 7) and on how difficult it would be for the surgeon to execute the plan. Finally, the setup with 6 electrodes presented in Figure 2 was selected for the treatment, with other treatment plans, including those with 4- and 5-electrode configurations (not presented) also prepared for back-up purposes. The final voltages and predicted currents, as well as actually delivered voltages and currents are shown in Table I with an overlay of the computational model and MRIs of the patient anatomy shown in Figure 3A. Two electrodes were positioned centrally in the tumor in order to provide sufficiently high electric field in the center of the tumor. The other 4 electrodes were positioned around the tumor in the normal liver tissue in order to provide the treatment of safety margins. These peripheral electrodes were positioned approximately 1-2 mm away from the tumor tissue.

Treatment Procedure

Identification of Metastasis and Preparatory Procedures Needed Before Delivery of Electric Pulses: During the operation in general anesthesia (December 2009) the left liver was mobilized, so that electrochemotherapy could be performed. The exact location of the tumor as well as the location of needle insertion according to the treatment plan was intraoperatively verified by US. Insertion of the needle electrodes was attempted to be as close as possible according to the treatment plan. The exact location of the electrodes was determined and was found later on to be in close match to the predetermined locations (Figure 2). Electrodes were inserted into the tumor and around it without any problem and without injury of any major blood vessel (Figure 3B).

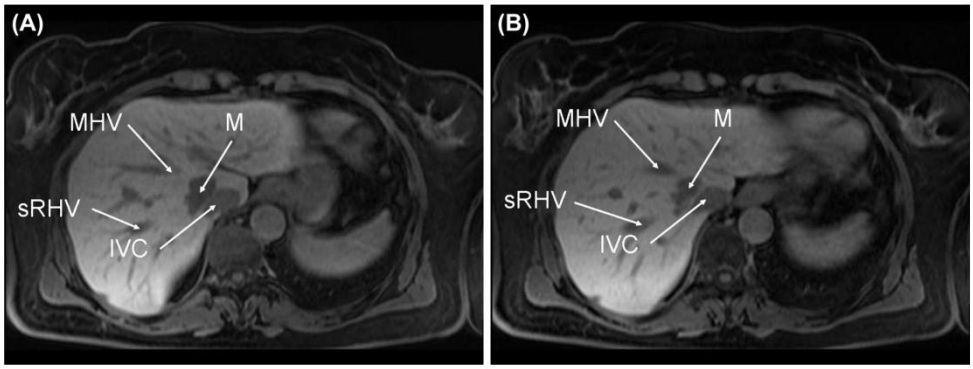

Figure 1: Axial T1W MRI image, showing a hypointense lesion (M) in between IVC and sRHV and MHV, in late liver phase, 20 min post Gd-EOB-DTPA. Images **A** and **B** are two consecutive images in 5 mm slice thickness.

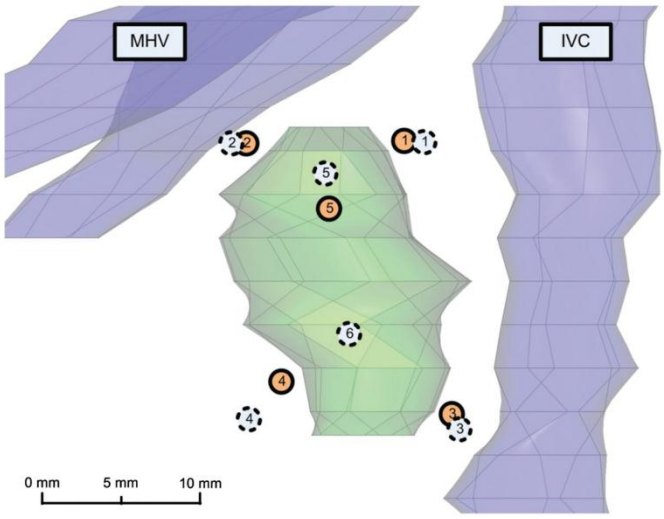

Figure 2: Design of treatment plan. The Figure shows the location of the tumor (green color) between the IVC and main hepatic veins (MHV, IVC, blue color). The solid circles represent electrode locations according to the original treatment plan and the dashed circles represent reconstructed electrode positions achieved *in situ*. The electrode 6 is in the same location in both cases.

After insertion into tissue the electrodes were connected to the Cliniporator *VITAE*, with special attention to correct wiring between the electrodes and the appropriate channel ports on the Cliniporator *VITAE*. Thereafter bleomycin (15000 U/m^2 of the patient) was injected intravenously in bolus. After 8 minutes, the time needed by the circulating bleomycin to reach the pharmacological peak in the tumor, the preparations for delivery of electric pulses were completed.

Delivery of Electric Pulses: The generic electroporation procedure is described first. The delivery of electroporation pulses is always preceded by a sequence of low-voltage pre-pulses.

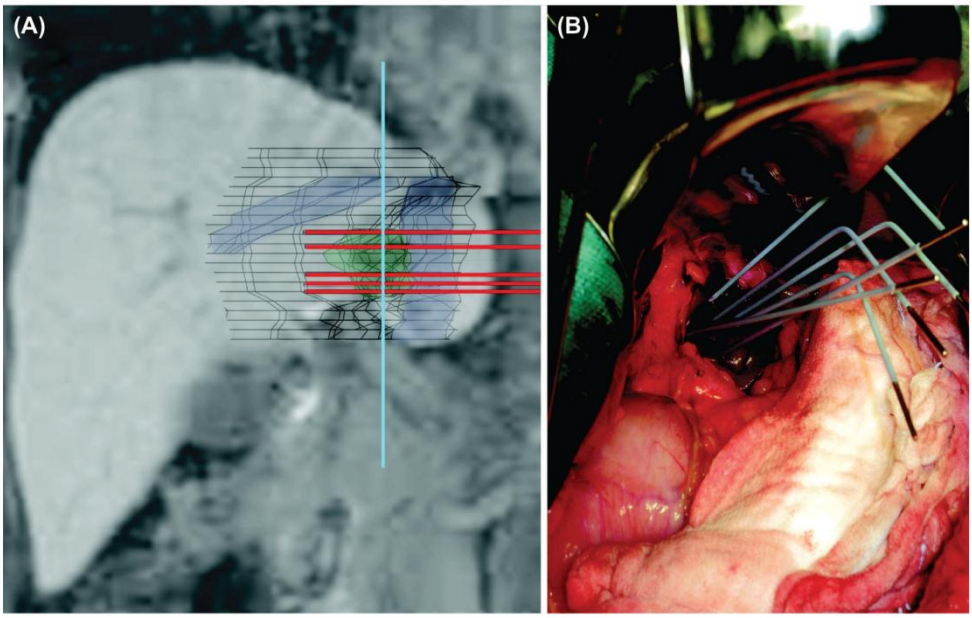

Figure 3: **A**: Overlay of the computational geometry and patient's anatomy. The red lines represent the direction of insertion of the electrodes, while the blue line represents the cross-section of Figure 2. **B**: Photograph of the surgical setup with electrodes penetrating into the tumor is clearly seen (cables not connected).

One pre-pulse is delivered to each pair of electrodes which is later to be used for actual electroporation according to the treatment plan. The purpose of pre-pulses is to verify the connections between the electrodes and Cliniporator *VITAE* outputs and also to predict (based on the current measured at low voltage) the current levels for the imminent electroporation pulses. For any pair of electrodes for which either a poor connection is found or the predicted current level exceeds 50 A (upper limit of the Cliniporator *VITAE*), the delivery of electroporation pulses is automatically suspended. Immediately after completion of the pre-pulse sequence, the high-voltage electric pulses, 8 per electrode pair, are delivered synchronized with the ECG (see the following subsection for details). The Cliniporator *VITAE* automatically suspends the delivery of electroporation pulses for: a) electrode pairs for which the pre-pulse sequence resulted in invalid values (current too low or too high); and b) for electrode pairs for which the actual current measured during electroporation itself exceeds the upper limit of 50 A, even though the predicted value based on the pre-pulse was below this limit. This completes the electroporation procedure. However, if needed, the procedure must then be repeated for all electrode pairs for which the delivery was suspended. Depending on the reason of suspension, the connections between the electrodes are checked and/or parameters of electroporation pulses are adjusted (voltage must be lowered if either the predicted or actual current exceeded 50 A). The whole electroporation procedure is then repeated just for the remaining electrode pairs. Sometimes more than one repetition may be required.

In our case, in the original treatment plan, 72 pulses were planned. However, a total of 105 electroporation pulses were delivered, because the electroporation procedure had to be repeated 5 times to complete delivery of all pulses. The larger number of actually delivered electroporation pulses is explained in the discussion. The entire electroporation procedure was finished 23.5 min after injection of bleomycin, *i.e.* within 15 minutes.

ECG Synchronization: The triggering of electric pulses was synchronized with ECG signals, through the ECG triggering device AccuSync, as described in previous section. The Cliniporator *VITAE* delivered one electroporation pulse per valid trigger pulse, which means that there was one pulse per heartbeat delivered. Whenever there was a transient loss of the ECG signal (due to ECG artifacts caused by muscle contractions after the previous pulse), the Cliniporator *VITAE* temporarily suspended pulse delivery until ECG and valid trigger pulse sequence were restored. The subsequent analysis of ECG signals showed that the synchronization procedure implemented in the Cliniporator *VITAE* resulted in safe and uneventful delivery of the pulses since all pulses were delivered outside the vulnerable period of the ventricles.

The preliminary evaluation of ECG signals recorded during the entire surgical procedure also revealed no heart arrhythmias or any other pathological morphological changes in any of the recorded signals either during or immediately after the application of pulses. However, some transient and statistically significant changes in the duration of some intervals in the PQRST complex were observed during the heartbeats coinciding with the delivery of electroporation pulses. Currently the practical significance of these changes (if there is any) is not clear and can only be elucidated when data from more patients treated in similar conditions become available.

Safety of Treatment

During and after the electrochemotherapy, there were no adverse effects which could be attributed to the procedure itself. Postoperative course was uneventful and the patient was released from the hospital on day 10.

Post Treatment Follow-up and Treatment

Two months after electrochemotherapy CT was performed. In the paracaval region toward the Sg 1 there was a homogeneous hypodense ovoid lesion (30 mm × 15 mm) without any new lesions (Figure 4).

There was no change in size of the metastasis treated with electrochemotherapy, however, CT image showed that margins were blurred, which demonstrated that treatment had some effect (Figure 4). At that time it was unclear what kind of the effect it was. The patient was suggested another exploration, which was accepted and the operation was performed three months after the first one. Intraoperative US examination showed hypo-echogenic changes, probably caused by necrosis. We mobilized left and right hemiliver and resected the part of paracaval region with Sg 1, preserving the main

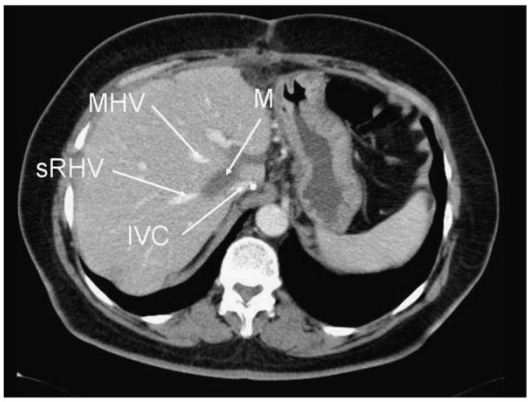

Figure 4: Axial CT image taken before the metastasis (M) was removed shows homogenous hypodense lesion in paracaval region toward Sg 1 (30 mm × 15 mm), with no signs of any new lesion.

hepatic veins and IVC (Figure 5). Using ultrasound dissector the Sg 1 was easily resected from sRHV and common trunk. The walls of both vessels were firm and not as tender as usually. There was very little blood loss and no transfusion was needed during and after the operation.

Gross pathological examination of the excised specimen showed oval, relatively sharply demarcated area of amorphous and yellowish tissue in the liver parenchyma. Histologically, complete necrosis was found and, in the vicinity of it, focal necrosis of the narrow zone of the liver parenchyma was present. In the excised lesion there was no viable tumor tissue. Taking into account that etiology of necrosis can be different, immunohistochemical staining for CK20, CEA and Hepat was performed. CK20 is an intermediate filament (part of cytoskeleton) mainly expressed in gastrointestinal type epithelia and carcinomas deriving from it. CEA is found in a large variety of carcinomas of gastrointestinal, respiratory and genitourinary tract while Hepat stains normal human hepatocytes. Although CK20 and CEA are not entirely specific for colorectal carcinoma, vast majority of them are strongly positive for both markers. In our case, necrotic tissue was immunohistochemically positive for CK20 and CEA, and negative for Hepat in contrast to vital liver parenchyma which was positive for Hepat and negative for CEA and CK20 (Figure 6). Findings like that support that necrosis arose from the metastatic carcinoma. Although it was not possible to discriminate between necrosis induced by ECT or chemotherapy only, there was indirect evidence of the effect of electrochemotherapy and that is the presence of necrosis of the narrow zone of the liver parenchyma surrounding the tumor. On the border between necrotic tumor tissue and vital liver parenchyma, focal proliferation of fibroblastic tissue with some chronic inflammatory reaction, foamy macrophages and pigmentophages were observed. In the remaining liver parenchyma slight portal fibrosis was present.

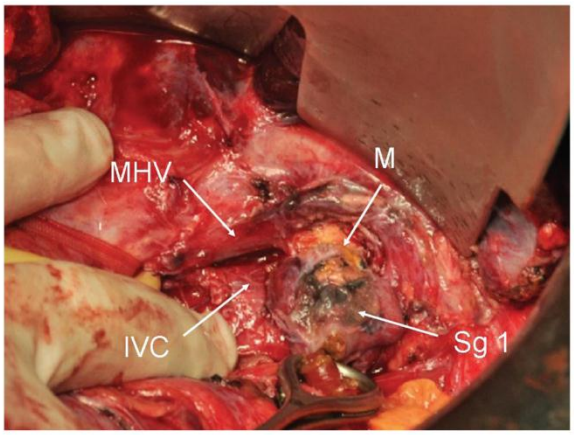

Figure 5: Resection of Sg 1 with common trunk and MHV exposed: Necrotic metastasis (M) is visible in Sg 1, close to the MHV and IVC.

Discussion

The results of the study show that electrochemotherapy on liver metastases can be performed safely and effectively. Treatment procedure for electrochemotherapy of liver metastases is presented and described on a patient with CRLM in paracaval region extending to Sg 1. This single metastasis in difficult location was successfully treated, as evident by histological examination of the removed metastasis after the second operation. The described procedure demonstrates its complexity, where several specific steps have to be taken in consideration; exact treatment planning for electrode positioning and the delivery of electric pulses, positioning of the electrodes before injection of bleomycin, as well as synchronization of electric pulses delivery with ECG for safety reasons. The patient is disease free 16 months after the electrochemotherapy procedure.

Radical removal of any malignant tumor from the paracaval region and Sg 1, close to the main hepatic veins inflow to IVC is challenging. In the presented case, the radical resection of the metastasis would have been potentially possible by doing right trisectionectomy leaving probably too little liver remnant (lateral part of the left liver). Considering the specific location, RFA would not be effective because of the cooling effect of the veins. All possibilities of treatment had been presented to the patient who decided to be treated with electrochemotherapy. The treatment was performed following very closely the treatment plan, without post-treatment side effects.

Treatment planning is needed for the exact positioning of the electrodes, in order to predict successful electroporation of the whole tumor mass. As it is known, two conditions have to be met for effective electrochemotherapy: presence of the drug during the electroporation in the tissue and electroporation of the whole tumor mass. Based on the presumption that intravenous drug administration would adequately deliver bleomycin to the liver metastasis, emphasis was put on electroporation treatment plan. Several treatment plans were prepared, in order to satisfy all possible situations that might have occurred (7). According to the numerical model, it was decided that one or two electrodes should preferentially be inserted in the tumor to improve the electric field distribution and guarantee complete tumor coverage. This was reported previously (7), and we have shown that positioning of electrodes outside the tumor is very sensitive to errors in electrode placement and differences in tissue conductivities between the target (tumor) and surrounding tissue (8).

Despite the effort to precisely follow the treatment plan for the positioning of the electrodes, the final positions of the electrodes did not match the original treatment plan exactly. These deviations from the original treatment plan (see Figure 2 and Table I) were considered in the post-surgery numerical

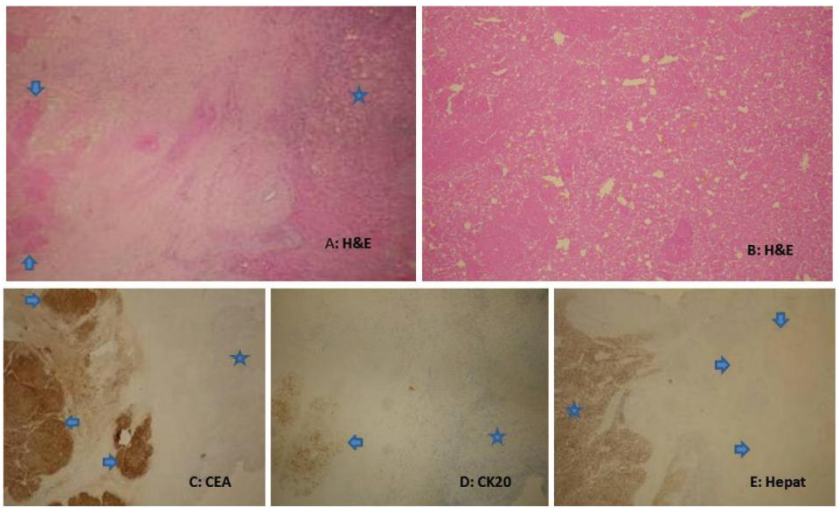

Figure 6: **A**: H&E, 4x; complete necrosis of the tumor tissue (arrows) and vital liver parenchima (star). **B**: H&E, 10x; completely necrotic tumor tissue. **C**: IHC CEA 4x; CEA positive staining in the necrotic tumor tissue (arrows) and negative in the vital liver parenchyma (star). **D**: IHC CK20 4x; CK20 positive staining in necrotic tumor tissue (arrow) and negative in vital liver tissue (star). **E**: IHC Hepat 4x; positive staining for Hepat in the liver (star) and negative in the necrotic tumor tissue (arrows).

evaluation that used the same numerical model as in treatment planning. The calculations showed that the treatment plan was still followed closely enough and that the electric field distribution was adequate for the procedure, *i.e.* the entire tumor volume was exposed to the electric field above 450 V/cm, with 90% being higher than 900 V/cm.

In the delivery of pulses between electrodes 1-5, 2-5, 3-6 and 4-6 (see Table 1) the originally planned amplitude of voltage for pulses could not be used due to excessive current values experienced during the first attempt to deliver the pulses (more than 50 A – a limitation of the Cliniporator *VITAE* device). Therefore the amplitude was lowered and a greater number of pulses at decreased voltage were delivered. It is known that electroporation can be effectively carried out (and the desired effect achieved) if a weaker electric field is used but with a greater number of pulses (23, 24). For this reason the number of pulses delivered to some of the electrode pairs was increased.

In all reports on clinical use of electrochemotherapy for treatment of cutaneous or subcutaneous malignant tumors the method has been described as completely safe. No serious side effects for the patient have ever been reported. The minor side effects reported in the literature include localized transient lesions in normal tissue in immediate vicinity of the treated region and the acute pain associated with contraction of skeletal muscles in vicinity of the electrodes which was caused either by direct electrical stimulation of the muscles or of the nerves innervating these muscles (25-27). The acute pain is the reason for use of either local or general anesthesia (depending on the location and number of tumors to be treated) during treatment of cutaneous or subcutaneous tumor lesions. Such electrochemotherapy treatment was shown to have no effect on the function of the heart apart from a transient and mild tachycardia attributed to anxiety of the patient in case of local anesthesia (10). This result was not surprising taking into account the high level of treatment localization (the electrodes are positioned close together and far away from the heart) and the very short duration of electric pulses. However, according to the results of the study on numerical calculations of electric field and current distribution for a tissue model it may be theoretically possible to affect functioning of the heart even in case of subcutaneous tumors located on the chest close to the heart and for deep insertion of needle electrodes (*e.g.* approximately 4 cm) (10). Under such extreme conditions the threshold value of current for induction of ventricular fibrillation (set at 100 mA for the given duration of electric pulses) could be exceeded (10). Furthermore, with recent development of new electrochemotherapy modalities for treatment of internal tumors using surgical procedures or endoscopic routes (28) to gain access to treatment area, could result in in the treated region located in close proximity of the heart. Due to the absence of protective barrier of the skin and relatively large electrical conductivity of internal tissues and organs the electrical current delivered during electrochemotherapy using invasive access, can propagate through a larger volume of

tissue surrounding the treated region. Therefore, an increased probability for electroporation pulses interfering with the heart function is present. In recently published studies on non-thermal irreversible electroporation, different minor and major hemodynamic and cardiologic changes due to unsynchronized irreversible electroporation pulse delivery were reported, such as systolic hypertension, supraventricular tachycardia, ventricular tachycardia with pressure drop, ventricular fibrillation, ST segment elevation and changes in T wave (11, 29). Deodhar *et al.* (11) showed that unsynchronized irreversible electroporation pulses delivered at less than or equal to 1.7 cm from the heart provoked fatal events (such as ventricular fibrillation) whereas pulses delivered more than 3 cm from the heart did not provoke any changes from baseline ECG (11). On the other hand, they reported that synchronized irreversible electroporation did not provoke any (fatal or minor) events at more than 1.7 cm distance from the heart. In the case reported here, the electroporation pulses were delivered at a location more than 10 cm away from the heart. Regardless of an unlikely event of serious consequences (such as induction of various arrhythmias or, in worst case, ventricular fibrillation), the synchronization of electroporation pulse delivery with the cardiac rhythm should be a prerequisite step for treatment of tumors in all internal organs, and especially those in close vicinity of the heart, to maximize the safety of the patient. The synchronization algorithm currently implemented in Cliniporator *VITAE* device coupled with the external triggering device AccuSync proved to be effective in preventing external stimulation of the heart during the so-called vulnerable period of the ventricles. As a result all electroporation pulses in our study were delivered outside the vulnerable period and no heart arrhythmias or any other pathological morphological changes were observed.

The safety of the treatment was demonstrated also by absence of side effects during and after electrochemotherapy. The use of electrochemotherapy did not extend hospital stay. Electrochemotherapy was performed in the vicinity of the big blood vessels (IVC, MHC, RHV), which may pose a specific problem. The electrodes are 1.2 mm in diameter and puncturing the vessels, specifically after retracting the electrodes may induce bleeding. However, also in this case, where electrodes were positioned nearby or even in or through the vessels no adverse events were recorded. No bleeding of the tissue after retraction of the electrodes was noticed. In the case of bleeding, the electrodes may be used as electrocoagulation tip by bringing them in contact with electrical surgical knife. This also has a preventive effect against bleeding. The mechanisms of action of electric pulses on vessels are known, for normal and tumor vessels (30-32). Electroporation induces immediate vasoconstrictive effect on vessels that is gradually released after a few hours. This immediate effect can be continued by vascular disrupting effect when the drug is present leading also to cytotoxicity of endothelial cells and abrogation of perfusion for a long period of time. This vascular disrupting effect of electrochemotherapy may add substantial part to overall effectiveness of electrochemotherapy in treatment of well vascularized tumors (31). However, there was no damage observed on normal liver tissue in our case. The results are in agreement with the results on liver tissue with irreversible electroporation where safe use of electroporation on bigger vessels in the liver was described (33).

Based on histological analysis, electrochemotherapy treated metastasis underwent complete necrotization within two months after the treatment. However, we are aware, that this patient was not treated with electrochemotherapy solely. After the metastasis was diagnosed, the patient was treated with systemic chemotherapy (XELOX + bevacizumab), and 5 weeks later received electrochemotherapy; so it is hard to put all credits for good treatment result to electrochemotherapy only, although chemotherapy was discontinued 5 weeks before electrochemotherapy. Although full responses to systemic chemotherapy are rare, they do occur, but histologically these metastases tend to develop central fibrosis (34). The usual responses to electrochemotherapy are non-necrotic, however also necrotic responses were recorded, as in the case of treatment of brain metastases (35, 36). To clarify and evaluate the treatment effectiveness on larger number of patients, there is an ongoing phase I/II clinical trial at the Institute of Oncology Ljubljana. The trial is designed to evaluate the effectiveness, safety and toxicity of the electrochemotherapy with bleomycin in treatment of the liver metastases originating from colorectal cancer. After recruiting a sufficient number of patients, we will have a much clearer picture about effectiveness of electrochemotherapy in treatment of liver metastases. Therefore, we must await further experience with this technique to get proof of clinical efficacy.

In case of encouraging results of the on-going clinical trial, further clinical trials performed on other liver tumors or liver metastases will be performed. With well elaborated and tested treatment planning system even percutaneous treatment of tumors using a guided system could be performed, that would be considerable improvement over invasive procedure described here (37).

In conclusion, electrochemotherapy proved to be feasible technological approach for treatment of liver metastases. Due to absence of side effects and the first reported complete destruction of the treated tumor, electrochemotherapy in treatment of liver metastases proved to be safe with good treatment effectiveness even in a difficult-to-reach location.

Conflict of Interest

We certify that regarding this paper, no actual or potential conflict of interest exists; the work is original, has not been accepted for publication nor is concurrently under consideration elsewhere, and will not be published elsewhere without the permission of the Editor and that all the authors have contributed directly to the planning, execution or analysis of the work reported or to the writing of the paper.

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PAPER V

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An algorithm for synchronization of *in vivo* electroporation with ECG

B. MALI†, T. JARM†, F. JAGER‡ and D. MIKLAVČIČ†*

†Faculty of Electrical Engineering, University of Ljubljana, Tržaška 25, SI-1000 Ljubljana, Slovenia
‡Faculty of Computer and Information Science, University of Ljubljana, Tržaška 25, SI-1000 Ljubljana, Slovenia

The combined treatment of tumours in which delivery of a chemotherapeutic agent is followed by high voltage electroporation pulses has been termed electrochemotherapy. The electrochemotherapy of tumours located relatively close to the heart muscle can lead to fibrillation of the heart, especially if electroporation pulses are delivered in the vulnerable period of the heart or in coincidence with heart arrhythmias. We built an electroporation pulse delivery algorithm that enables safer use of electrochemotherapy. The algorithm is designed to deliver pulses outside the vulnerable period and to prevent pulses from being generated in the presence of heart arrhythmias. We evaluated the algorithm's performance using records of the Long-Term ST Database, thus simulating real-world conditions. The results of the evaluation, a sensitivity of 91.751%, a positive predictivity of 100.000% and a delivery error rate of 8.268% for electroporation pulse delivery (medians), suggest that the algorithm is accurate and appropriate for application in electrochemotherapy of tumours regardless of tumour location.

1. Introduction

The combined treatment in which delivery of a chemotherapeutic drug is followed by application of high voltage electric pulses locally to the tumour has been termed electrochemotherapy. The effect of local electropermeabilization of the cell membrane—also termed electroporation (EP)—enables entry of drug molecules into the cells and hence greater effectiveness of the tumour treatment. Electrochemotherapy has been successfully used for treatment of various cutaneous and subcutaneous tumours in different animal tumour models and in humans [1–4]. In these studies, a typical electrochemotherapy protocol involved eight EP pulses with amplitude approximately 1000 V, duration 100 μs, repetition frequency 1 Hz, and inter-electrode distance 8 mm. Beside this protocol, other protocols for delivery of EP pulses are either already being used or are expected to be developed and used in the future. For example, the protocol involving eight EP pulses at repetition frequency of 5 kHz has been suggested and is currently replacing the 1 Hz protocol due to a lesser discomfort and pain inflicted in patients [5]. Moreover, pulses of a much longer duration (on the order of milliseconds) are used for electrogene therapy [6]. Another successful protocol for electrogene transfection relies on a combination of high voltage EP pulses with very long low voltage electrophoretic pulses (amplitude 50–100 V, duration 100 ms) in order to optimize gene transfer [7,8].

In spite of the increasing clinical use of electrochemotherapy this treatment has some minor side effects including transient lesions in areas in direct contact with the electrodes [9] and acute localized pain due to contraction of muscles in vicinity of the electrodes [2]. This induced contraction would become a problem if it were provoked in the heart muscle [10]. There is very little chance and absolutely no practical evidence that any electroporation protocol mentioned above could interfere with functioning of the heart when applied to cutaneous and subcutaneous tumours. However, a need for palliative treatment of internal tumours has emerged lately and treatment of internal tumours located close to the heart muscle would increase the probability of EP pulses interfering with the heart. The most dangerous possible interference is induction of ventricular fibrillation [10–12]. This issue is becoming increasingly important because new applications using endoscopic or surgical means to access internal tumours are being developed [13]. An algorithm for synchronization of EP pulse delivery with ECG to

*Corresponding author. Email: damijan@svarun.fe.uni-lj.si

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maximize the safety for patients is needed before these applications can be realized.

Fibrillation of the heart can be induced if the amplitude of the externally applied electric pulses in a part of the heart is greater than the threshold level for fibrillation and if electrical stimulation is delivered during late atrial or ventricular systole—during the so-called vulnerable period [10,11,14]. For the ventricles, the vulnerable period is near the peak of the T wave and for the atria, it is probably in the S wave [15] (figure 1).

Although fibrillation can occur in normal and healthy hearts, it is more likely in hearts with structural or functional abnormalities [16]. Abnormalities of the heart rhythm (arrhythmias) are indicated by significant deviation of RR interval from its normal value [17]. During some arrhythmias the heart becomes significantly more susceptible to external stimuli due to a decreased threshold level for fibrillation. Therefore EP pulses coinciding with some arrhythmias could elicit fibrillation. This potential danger is most significant after the so-called premature response, the extrasystole [10].

In order to enable safer use of EP pulses during electrochemotherapy we developed an algorithm for synchronization of EP pulses with ECG. The algorithm allows EP pulses to be delivered only outside the vulnerable period of normal heartbeats (figure 1) and prevents the EP pulses from being generated in the presence of some common heart arrhythmias.

2. Methods

2.1. The algorithm

For application in electrochemotherapy the algorithm for QRS detection has to be simple enough for real-time realization, must enable early detection of QRS complex (i.e. detection based mainly on analysis of QR junction) and has to be able to distinguish well between normal and abnormal heartbeats. Based on the published algorithms [18] we developed a new algorithm that adequately fulfils these requirements. It is based on analysis of a single ECG lead at sampling frequency of 250 Hz and operates on individual signal samples in time-domain. It searches for the initial portion of the QRS complex, i.e. the ascendant QR junction slope and R wave peak, as early as possible prior to the vulnerable period, thus leaving enough time within QRS complex for electroporation pulse delivery.

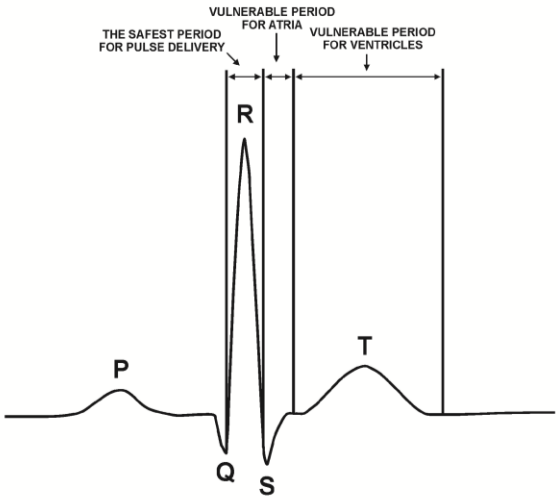

Figure 1. The vulnerable period for ventricles and atria.

During the electrochemotherapy protocol the patient is resting comfortably and thus the conditions for noise-free ECG signal prior to electroporation are fulfilled. Furthermore, the whole procedure of electrochemotherapy including the preparation of the patient is short (up to 10 minutes), while the electroporation procedure is even shorter (measured in seconds). The electrochemotherapy is performed only on patients without severe heart disease, so ECG signals without or with only minimal pathological changes can be expected. According to the electrochemotherapy protocol, there is currently no need for an algorithm for processing ECG signals containing distinctive noise and rapid changes of QRS complex morphology due to shifts of the mean electrical axis as a consequence of postural changes. To enable early detection of QRS complex an ECG lead with distinctive ascendant QR junction, high R wave amplitude and high dynamics within QRS complex in comparison to other parts of ECG signal is required. Typical standard ECG leads fulfilling these requirements include the chest lead V_4 and standard limb leads I and II. We use the term 'V_4-like' lead for all leads suitable for our application. A 'V_4-like' lead can easily be obtained in practice because it can be created by moving the ECG electrode to an arbitrary position, should none of the standard leads be appropriate.

The algorithm for synchronization of EP pulse delivery with ECG consists of two major components (the detection phase and the decision-making phase), which are preceded by the learning phase (figure 2).

2.1.1. The learning phase. Adequate functioning of the algorithm is based on three main architecture parameters estimated during the learning phase from the ECG signal: the average value of the combination of the first and second derivative of the ECG signal ($\bar{Y}_{12}$); the running average R wave amplitude ($\bar{R}$); and the running average RR interval ($\overline{RR}$). Inter- and intra-record variability of these three parameters is very common. Therefore the algorithm determines their initial values during the learning phase. During this phase, an essentially noise-free ECG signal without heart arrhythmias or other heart abnormalities is required for fast adaptation to the given signal characteristics.

During part I of the learning phase (a 20-s interval) the value of the parameter $\bar{Y}_{12}$ is determined. This interval is divided into 20 equal subintervals. Within each subinterval, the maximum value of the slope parameter Y_{12} (the combination of absolute values of first and second derivative of ECG signal) is determined using equations from [18]:

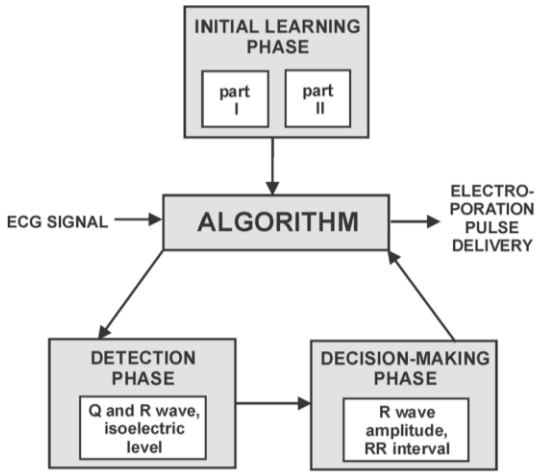

Figure 2. The structure of the algorithm.

$$Y_1(n) = \text{abs}[X(n+1) - X(n-1)], \quad (1)$$

$$Y_2(n) = \text{abs}[X(n+2) - 2 \cdot X(n) + X(n-2)], \quad (2)$$

$$Y_{12}(n) = 1.3 \cdot Y_1(n) + 1.1 \cdot Y_2(n), \quad (3)$$

where $X(n)$ is the sequence of the ECG signal's samples. Normally the maximum values of Y_{12} should be found on QRS complexes. The five largest and five smallest Y_{12} values thus found are omitted and the average value ($\bar{Y}_{12}$) is calculated from the remaining 10 values.

During part II of the learning phase the algorithm detects consecutive QRS complexes by using the threshold value Y_{th} (based on $\bar{Y}_{12}$) and estimates the other two parameters ($\bar{R}$ and $\overline{RR}$) with procedure described in next section. The threshold value Y_{th} is taken as one seventh of $\bar{Y}_{12}$; this ratio was set empirically. The initial average R wave amplitude ($\bar{R}$) and RR interval ($\overline{RR}$) are calculated based on the latest 16 R wave amplitudes and the latest 8 RR intervals respectively. These averages, which are constantly being updated, are used in ECG analysis later on for calculation of several threshold parameters. These threshold parameters and their roles are described in the subsection on decision-making phase.

2.1.2. The detection phase. The algorithm for EP pulse delivery is based on accurate QRS complex detection, which is often difficult to achieve, since various sources of noise contamination and morphological differences in the ECG waveforms are frequently encountered [19]. The slope of the QR or RS interval of the QRS complex is a popular signal feature used to locate the QRS complex in many QRS detectors [18,20–22]. A real-time derivative algorithm that provides slope information is straightforward to implement but a slope alone is insufficient for accurate QRS complex detection. To achieve a reliable QRS detector performance, additional parameters often have to be extracted from the signal such as the R wave amplitude, the width of the QRS complex, the RR interval, or the QRS energy [22].

Our QRS detector is an adaptation of the detector described in [18]. In order to improve the performance of the algorithm and to assure clear distinction between normal and abnormal individual heartbeats, we included additional signal features into consideration: the QR interval, the R wave amplitude and the RR interval. For implementation of such a detector the peaks of Q and R waves and the isoelectric level must be extracted from the ECG signal.

First, in order to locate the ascendant QR slope and R peak of the QRS complex, the algorithm searches for seven successive samples (set empirically and valid for a sampling frequency of 250 Hz) for which Y_{12} is greater than or equal to Y_{th} (figure 3). The sign of the first derivative must be positive in the first five samples of this set of seven, negative or equal to zero in the sixth sample and negative in the seventh sample. If all these conditions are fulfilled, it is very likely the algorithm has located the R wave. All together 11 successive signal samples (marked with a 'star' symbol in figure 3) are used for the procedure of finding Y_{12} in seven successive samples.

Second, the peak of R wave is sought for. The peak of R wave corresponds to the sample with maximum amplitude among the 11 samples used so far (figure 3). Third, the Q peak is detected. The algorithm calculates the first derivative backwards from the R peak for 80 ms until it finds four successive signal samples among which the first (counted from the left to the right) has either a negative or zero first derivative and the other three have a positive first derivative (figure 3). The Q peak thus corresponds to either the first or the second of these four samples. After this, the QR interval can be defined. Fourth, the correctness of the R wave location is further assured by comparing the QR interval to typical normal QR interval, which is approximately 0.03 s long [10]. If the current QR interval is not within the 0.02 – 0.10 s range (set empirically), the analysis of the current heartbeat is discontinued and the next normal heartbeat is sought for. The length of QR interval also helps in distinguishing the QRS complex from high frequency noise. Fifth, the flattest part of the PQ segment, termed the isoelectric level, is determined. The algorithm searches for the isoelectric level backwards from the Q peak but only up to 108 ms backwards from the R peak [23]. For each series of five successive samples within this interval the algorithm calculates the average amplitude value and the total deviation value. The average value of the samples that have the minimal total deviation from the average value is taken as the isoelectric level. Finally, the R wave amplitude can be calculated as the difference between the R peak value and the isoelectric level.

2.1.3. The decision-making phase. During the decision-making phase, the algorithm decides about delivery of the EP pulses with respect to deviations of R wave amplitude and heart rate (indicated by the RR interval) of individual heartbeats from average values of $\bar{R}$ and $\overline{RR}$.

If the value of amplitude of the current QRS complex is within $\pm$ 30% of $\bar{R}$ and if the value of the current RR interval is within – 7% to + 15% of $\overline{RR}$, the current QRS

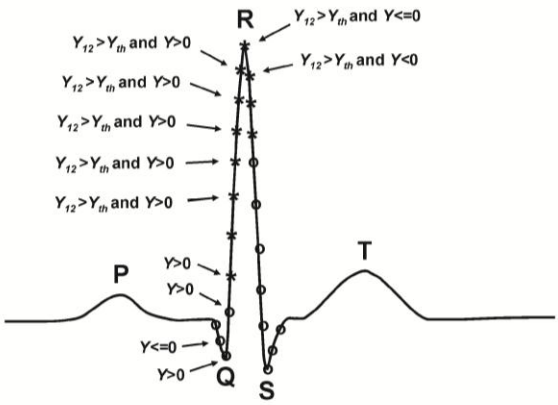

Figure 3. The conditions for detection of R and Q wave. For R wave detection the value of Y_{12} for seven successive samples has to be larger than the threshold value Y_{th} and at the same time the value of first derivative (Y) must be positive for the first five, negative or zero for the sixth, and negative for the seventh sample. Eleven successive samples (marked with a 'star' symbol) are used for this procedure. For Q wave detection the first value of Y among four successive Y values must be negative or zero and the remaining three Y values positive.

complex is considered non-pathological, i.e. normal. Therefore, the algorithm would deliver the EP pulse. These threshold values for EP pulse delivery have to follow slow morphology changes of the signal, which occur normally in any ECG signal. Therefore $\overline{R}$ and $\overline{RR}$ values are calculated as the moving average of the 16 and eight most recent values, respectively, that also fall within the following threshold values for updating. If the value of amplitude of the current QRS complex is within $\pm$ 40% of $\overline{R}$ and if the value of the current RR interval is within $\pm$ 25% of $\overline{RR}$, the current averages $\overline{R}$ and $\overline{RR}$ are updated. The expression of all threshold values in terms of percentages of $\overline{R}$ and $\overline{RR}$ that we report here are based on empirical evaluation of the algorithm's performance.

We incorporate only eight RR intervals in the running average $\overline{RR}$ (in comparison with 16 R wave amplitudes used in the running average $\overline{R}$) because the RR interval is expected to be less steady than the R wave amplitude in normal heartbeats. Therefore it is possible to account for relatively rapid but normal changes in the rhythm of the heart. This feature is of great importance for proper distinguishing between normal heartbeat intervals and heart arrhythmias. The threshold values for updating are set wider apart than the threshold values for the EP pulse delivery. The reason for this is that we must be extremely cautious about the EP pulse delivery and thus be convinced that we are really dealing with a non-pathological heartbeat. While inclusion of an abnormal heartbeat in calculation of running averages $\overline{R}$ and $\overline{RR}$ is not critical (e.g. one abnormal value among 15 or seven normal ones, respectively), delivery of an EP pulse in such conditions could be dangerous.

The possibility of the onset of various arrhythmias is of predominant concern when deciding whether to deliver an EP pulse or not. Therefore, even when our QRS detector finds a QRS complex, the algorithm would not allow for the EP pulse delivery if the current RR interval significantly deviates from $\overline{RR}$. The allowed deviation of current RR interval is -7% to $+15\%$ from $\overline{RR}$. The lower threshold was chosen because extrasystoles can occur as little as 7–10% prematurely [10] and we absolutely want to avoid EP pulse delivery in the case of extrasystoles. Initially the upper threshold was set to $+7\%$ but this resulted in an unacceptable decrease in the number of delivered pulses. Since the heartbeats delayed for more than 7% are not problematic for our application, we found the upper threshold of $+15\%$ to be appropriate.

2.2. Evaluation of the algorithm

2.2.1. ECG database. We evaluated the performance of our algorithm using selected records of the Long-Term ST Database (LTST DB) [24]. The LTST DB contains approximately 24-hour long ambulatory Holter records reflecting the real-world clinical environment with all heartbeats classified by experts. Therefore all usual daily activities of the patient are reflected in ECG signals, which also contain abnormal heartbeats of various pathological backgrounds.

Since many records are not 'V_4-like' and since we do not expect extreme conditions with respect to abnormal heartbeats, nor severe levels of noise in the input signals during electrochemotherapy protocol, we selected only 42 records from the pool of 86 records of the LTST DB. First, we excluded the records containing QRS complex conduction changes (s20531, s20541, s20551, s30721) and unreadable intervals (s20291, s20561, s20571, s20601, s20621, s30761, s30801). The electroporation pulse delivery would not be started or would be terminated under such conditions in clinical practice. Then we excluded non-'V_4-like' ECG signals (s20191, s20221, s20251, s20301, s20311, s20341, s20391, s20431, s20461, s20481, s20581, s20591, s20611, s20641, s20651, s30661, s30681, s30771, s30781). During the electrochemotherapy, a requirement for accurate electroporation pulse delivery is a suitable QRS complex morphology. Then, with respect to graphic trends of diagnostic and morphology parameters of the database [24], we excluded the records containing significant axis shifts and consequently rapid significant changes in the QRS complex morphology (s20051, s20201, s20271, s20272, s20273, s20274, s20331, s20501, s30731, s30732), and records containing considerable noise intervals (s20041, s20511, s20521, s20161). The remaining records and ECG leads used to evaluate the performance of our algorithm are evident from table 1.

In the process of developing the algorithm we used two two-hour-long sections from the beginning of two ECG signals from the LTST DB. The first section from signal s20011 features mostly normal heartbeats and only an insignificant number of pathological heartbeats (see table 1). The second section from signal s20101 includes a lot of

Table 1. ECG signals from the LTST DB, ECG lead used, number of heartbeats of particular type, results of QRS detection and results of EP pulse delivery†.

Signal name	Signal number	Lead name	*N*	*V*	*S*	N_d	*TP*	*FN*	*FP*	*Se (%)*	*+ P (%)*	*DER (%)*	N_p	TP_p	FN_p	FP_p	V_p	S_p	Se_p *(%)*	$+ P_p$ *(%)*	DER_p *(%)*
s20011	0	ML2	100028	2	23	100014	99883	131	0	99.869	100.000	0.131	99989	97201	2788	1	0	1	97.212	99.999	2.789
s20021	1	V_4	88755	138	69	88923	88589	334	1	99.624	99.999	0.377	88716	85642	3074	1	0	1	96.535	99.999	3.466
#s20031	1	*x*	107069	1	2430	109460	109201	259	1	99.763	99.999	0.238	107028	101540	5488	1	0	1	94.872	99.999	5.129
s20061	0	ML2	117114	776	4	117873	117069	804	0	99.318	100.000	0.682	117092	99562	17530	0	0	0	85.029	100.000	14.971
s20071	0	ML2	85745	5	0	85714	85437	277	0	99.677	100.000	0.323	85701	82602	3099	0	0	0	96.384	100.000	3.616
#s20081	0	ML2	111662	1292	2	112936	1100	2806	1	97.515	99.999	2.485	111642	96589	15053	0	0	0	86.517	100.000	13.483
s20091	0	ML2	111565	0	5	111331	111321	10	0	99.991	100.000	0.009	111326	90499	20827	0	0	0	81.292	100.000	18.708
#s20101	0	ML2	70504	7081	48	77978	70357	7621	0	90.227	100.000	9.773	70849	60656	10193	1	0	1	85.613	99.998	14.388
s20111	0	ML2	85186	3	75	85218	84806	412	0	99.517	100.000	0.483	85139	83314	1825	0	0	0	97.856	100.000	2.144
s20121	0	ML2	84927	580	19	85490	84358	1132	0	98.676	100.000	1.324	84891	83361	1530	0	0	0	98.198	100.000	1.802
s20131	0	ML2	105765	495	33	106256	105338	918	0	99.136	100.000	0.864	105727	103984	1743	0	0	0	98.351	100.000	1.649
s20141	0	ML2	116625	0	49	116630	116577	53	0	99.955	100.000	0.045	116581	112825	3756	3	0	3	96.778	99.997	3.224
s20151	0	V_4	79492	166	6	79638	79437	201	0	99.748	100.000	0.252	79466	75596	3870	0	0	0	95.130	100.000	4.870
s20171	0	V_4	126515	9	2	126481	126173	308	0	99.756	100.000	0.244	126470	115118	11352	0	0	0	91.024	100.000	8.976
s20181	1	V_4	106836	125	15	106913	104140	2773	2	97.406	99.998	2.596	106772	93166	13606	0	0	0	87.257	100.000	12.743
s20211	0	ML2	99840	17	2	99793	99531	262	1	99.737	99.999	0.264	99773	81670	18103	0	0	0	81.856	100.000	18.144
s20231	0	ML2	103061	2	34	103057	100346	2711	0	97.369	100.000	2.631	103018	81352	21666	0	0	0	78.969	100.000	21.031
s20241	0	ML2	92424	6	3	92381	92151	230	0	99.751	100.000	0.249	92366	71851	20515	0	0	0	77.789	100.000	22.211
s20261	0	*x*	101491	746	71	102270	101508	762	0	99.255	100.000	0.745	101451	94723	6728	5	5	0	93.368	99.995	6.637
s20281	0	*x*	72989	0	87	73041	72979	62	0	99.915	100.000	0.085	72954	70920	2034	0	0	0	97.212	100.000	2.788
s20321	0	V_4	91679	201	49	91888	91507	381	3	99.585	99.997	0.418	91638	78369	13269	0	0	0	85.520	100.000	14.480
s20351	0	*x*	118847	796	9	119607	118736	871	0	99.272	100.000	0.728	118802	116595	2207	0	0	0	98.142	100.000	1.858
s20361	1	*x*	105658	11	18	105650	105169	481	0	99.545	100.000	0.455	105621	102349	3272	1	0	1	96.902	99.999	3.099
s20371	0	*x*	95733	5	12	95710	95602	108	1	99.887	99.999	0.114	95693	92693	3000	0	0	0	96.865	100.000	3.135
s20381	0	*x*	102904	53	15	102924	94375	8549	1	91.694	99.999	8.307	102856	90754	12102	6	4	2	88.234	99.993	11.772
s20401	1	*x*	77269	61	3	77299	77160	139	0	99.820	100.000	0.180	77235	62700	14535	0	0	0	81.181	100.000	18.819
s20411	1	*x*	84385	23	299	84672	84543	129	0	99.848	100.000	0.152	84350	83133	1217	3	0	3	98.557	99.996	1.446
#s20421	1	*x*	87657	93	5211	92920	90719	2201	0	97.631	100.000	2.369	87614	79894	7720	3	0	3	91.189	99.996	8.815
#s20441	1	*x*	89666	3436	23	93078	88884	4194	0	95.494	100.000	4.506	89617	80678	8939	2	0	2	90.025	99.998	9.977
s20451	0	*x*	88006	3	46	88021	79336	8685	3	90.133	99.996	9.870	87972	71968	16004	1	0	1	81.808	99.999	18.193
s20471	0	V_4	115231	61	10	115258	115078	180	1	99.844	99.999	0.157	115187	107227	7960	0	0	0	93.089	100.000	6.911
s20491	0	V_4	96128	775	47	96911	92174	4737	9	95.112	99.990	4.897	96088	75055	21033	1	0	1	78.111	99.999	21.890
s20631	0	V_4	100030	0	6	99994	95897	4097	1	95.903	99.999	4.098	99988	83912	16076	0	0	0	83.922	100.000	16.078
s20671	1	V_5	98545	350	143	99040	98547	493	3	99.502	99.997	0.501	98547	90110	8437	16	0	16	91.439	99.982	8.578
s30691	1	A-S	101655	1	27	101644	98794	2850	2	97.196	99.998	2.806	101616	95399	6217	0	0	0	93.882	100.000	6.118
s30701	2	A-I	107061	0	17	107036	104129	2907	0	97.284	100.000	2.716	107019	101684	5335	0	0	0	95.015	100.000	4.985
#s30711	1	A-S	134278	19041	10	153227	19040	134187	0	12.426	100.000	87.574	134176	18671	115505	0	0	0	13.915	100.000	86.085
s30741	0	E-S	123461	0	0	123419	123116	303	0	99.754	100.000	0.246	123419	119692	3727	0	0	0	96.980	100.000	3.020
s30742	0	E-S	113767	0	0	113696	113591	105	0	99.908	100.000	0.092	113696	109954	3742	0	0	0	96.709	100.000	3.291
#s30751	2	A-I	102514	3476	66	106017	102306	3711	1	96.500	99.999	3.501	102475	94341	8134	20	20	0	92.062	99.979	7.957
#s30752	1	A-S	114473	5991	81	120414	90237	30177	0	74.939	100.000	25.061	114342	78165	36177	53	53	0	68.361	99.932	31.686
s30791	1	A-S	100325	57	99	100433	88245	12188	0	87.865	100.000	12.135	100214	84629	15585	0	0	0	84.448	100.000	15.552

(*continued*)

Table 1 (*continued*)

Signal name	Signal number	Lead name	*N*	*V*	*S*	N_d	*TP*	*FN*	*FP*	*Se (%)*	*+P (%)*	*DER (%)*	N_p	TP_p	FN_p	FP_p	V_p	S_p	Se_p *(%)*	$+P_p$ *(%)*	DER_p *(%)*
Total	–	–	4216865	45878	9168	4270255	4026516	243739	31	–	–	–	4215116	3700143	514973	118	82	36	–	–	–
Min	–	–	70504	0	0	73041	19040	10	0	12.426	99.990	0.009	70849	18671	1217	0	0	0	13.915	99.932	1.446
25%	–	–	88982	2	6	92011	88331	237	0	97.218	99.999	0.244	88941	80090	3386	0	0	0	84.593	99.999	3.335
Median	–	–	100908	55	21	101039	97222	628	0	99.410	100.000	0.591	100833	87876	8047	0	0	0	91.751	100.000	8.268
75%	–	–	110441	559	62	110863	105296	2893	1	99.756	100.000	2.783	110252	98972	15452	1	0	1	96.761	100.000	15.407
Max	–	–	134278	19041	5211	153227	126173	134187	9	99.991	100.000	87.574	134176	119692	115505	53	53	16	98.557	100.000	86.085
Mean	–	–	100402	1092	218	101673	95869	5803	1	95.365	99.999	4.635	100360	88099	12261	3	2	1	88.419	99.997	11.584
St. dev.	–	–	14491	3228	873	15559	18177	20940	2	13.902	0.002	13.902	14461	17946	17996	9	9	3	13.869	0.011	13.870

†*N* = normal heartbeat; *V* = ventricular premature beat; *S* = supraventricular premature or ectopic beat; N_d = 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); *FP* = false positive for QRS detection (the number of false QRS detections); *Se* = sensitivity for QRS detection; *+P* = positive predictivity for QRS detection; *DER* = detection error rate for QRS detection; N_p = total number of normal QRS complexes (total number of possible delivered EP pulses), the sum of TP_p and FN_p; TP_p = true positive for EP pulses (the number of EP pulses delivered at correctly detected normal QRS complexes); FN_p = false negative for EP pulses (the number of correctly detected normal QRS complexes. where no EP pulse was delivered); FP_p = false positive for EP pulses (the number of EP pulses delivered in absence of correctly detected normal QRS complexes); V_p = number of EP pulses delivered within ventricular premature beat; S_p = number of EP pulses delivered within supraventricular premature or ectopic beat; Se_p = sensitivity for EP pulses; $+P_p$ = positive predictivity for EP pulses; DER_p = delivery error rate for EP pulses; # = ECG signals with relatively poor values of performance metrics due to the presence of many heart arrhythmias; *x* = lead of ECG record unknown.

heart arrhythmias (see table 1) and other heart rate-related changes. Since the sections used for the development of the algorithm were only two-hour parts of the recorded signals, we included whole signals in the evaluation of the algorithm as well.

2.2.2. Performance metrics. For evaluation of QRS complex detection we calculated the following scores for each record: N_d, TP, FN and FP (for definitions see table 1). Based on these scores obtained with a beat-by-beat comparison of the results of our algorithm with true human-expert annotations of the heartbeats defined in the LTST DB, we calculated standard performance measures of the algorithm: sensitivity (Se), positive predictivity ($+P$) and detection error rate (DER) for QRS detection (equations (4)–(6), respectively). The performance measures for an ideal QRS detector would be $Se = 100\%$, $+P = 100\%$ and $DER = 0\%$.

$$Se(\%) = \frac{TP}{N_d} \cdot 100, \tag{4}$$

$$+P(\%) = \frac{TP}{TP + FP} \cdot 100, \tag{5}$$

$$DER(\%) = \frac{FP + FN}{N_d} \cdot 100. \tag{6}$$

For evaluation of EP pulse delivery we calculated the following scores for each record: N_p, TP_p, FN_p and FP_p (for definitions see table 1). Based on these scores and in absence of any standard performance metrics for EP pulse delivery, we calculated the performance measures analogous to QRS detection metrics: sensitivity (Se_p), positive predictivity ($+P_p$) and delivery error rate (DER_p) for EP pulses. The performance measures for an ideal algorithm for EP pulse delivery would be $Se_p = 100\%$, $+P_p = 100\%$ and $DER_p = 0\%$.

2.3. Programming

The algorithm itself and all routines for evaluation of performance were written in ANSI C programming language and implemented on a PC platform. In the future this will enable an easy translation of the algorithm into assembly language and its application in the existing microprocessor-driven instrument for clinical use of electroporation.

3. Results

The performance of the algorithm summarized in table 1 shows the ability of the algorithm to detect QRS and to deliver EP pulses correctly. Since data pertaining to individual records were not normally distributed, we provide a statistical summary of the results using both the mean and standard deviation and the median and quartile values. However, when we say 'on average' in the text we are always referring to median values, which are more representative of the middle of the sample and population than the mean values.

On average, the algorithm correctly detected 99.410% of all QRS complexes. The total number of erroneously detected QRS complexes was only 31, which is a very small number compared to the total number of QRS complexes (over 4×10^6). The detection error rate for QRS detection (DER) was 0.591% on average. Average positive predictivity for QRS detection ($+P$) was 100.000%.

On average, the algorithm correctly delivered EP pulses in 91.751% of normal QRS complexes. The value of delivery error rate for EP pulses (DER_p) was 8.268% and the value of positive predictivity for EP pulses ($+P_p$) 100.000% on average.

4. Discussion

The algorithm for synchronization of EP with ECG reliably detected QRS complexes in all 42 'V_4-like' signals from the LTST DB (see table 1). The algorithm allowed for EP pulse delivery for each correctly identified heartbeat if no abnormalities were detected. The performance of our algorithm (see §2.2.2) approached the ideal level at a degree similar to that of some other detectors with comparably simple algorithms [19,25]. The records with poorest results of our algorithm (large DER) contain very unstable R wave amplitudes, very unstable RR intervals and a transient appearance of high frequency noise with amplitudes similar to the R wave. At this preliminary stage of development our algorithm is not well suited to deal with signals that are largely nonstationary or have very low signal-to-noise ratio. However, due to its conservative nature the algorithm deals well with mildly nonstationary parts of the signal or transient onsets of noise contamination with low amplitudes, which were occasionally encountered in most of the signals used in evaluation of the algorithm. Moreover, many of the false negative detections (FN) were due to very strict requirements for no false positive detections (FP).

The algorithm could deliver the EP pulse either before vulnerable period or after it. Since the vulnerable period can sometimes be prolonged (e.g. after premature response) [10], it is obviously more reasonable to deliver the EP pulse before rather than after the onset of vulnerable period. Therefore, the most appropriate moment for EP pulse delivery is immediately after the QRS detection but still within the QRS complex. The delivery of EP pulses during vulnerable period of the atria does not present a serious threat for the patient's life. The haemodynamic effects of atrial flutter and fibrillation, which could be potentially caused by EP pulse delivery during vulnerable period for atria, are only slight and the patients are frequently unaware of these arrhythmias [10]. Bearing these facts in mind we can conclude that the time reserve for safe EP pulse delivery after the QRS detection and before the onset of vulnerable period for ventricles is approximately 60 ms and is long enough for safe EP pulse delivery even if we want to avoid the vulnerable period of atria as well.

Our main concern is to avoid, at any cost, delivery of EP pulses at the moments of potential danger for the patient. The algorithm fulfilled this requirement excellently as

indicated by practically ideal $+P_p$ values (table 1). We made our algorithm deliberately more conservative than would be necessary if the purpose was solely to detect QRS complexes. It is completely acceptable to miss some normal heartbeats (increase in FN and FN_p) as long as no EP pulse is delivered when it absolutely should not be ($FP_p = 0$!). Upon careful examination, we found 16 ECG signals in which the FP_p was not zero. The erroneously delivered pulses (there were 118 such pulses in comparison to approximately 3.7×10^6 correctly delivered EP pulses) coincided with appearance of some extrasystoles of either supraventricular or ventricular origin. The reason for FP_p is mainly in morphology (e.g. the heartbeats without distinct P wave) and the time of appearance of particular arrhythmias that are sometimes indistinguishable from the normal heartbeats.

In the ECG records from LTST DB all activities of a tested person during the day are reflected. Moreover, some of these ECG records contain numerous arrhythmias (see ECG signals marked with a 'hash' symbol in table 1). By including these records in our evaluation we actually tested the algorithm in conditions more severe than expected during clinical application of electroporation. The algorithm still worked well if we consider the number of FP_p that appear on arrhythmias in comparison to total number of arrhythmias.

Figure 4 is an example of the correct functioning of our algorithm on ECG signal containing ventricular extrasystoles. EP pulses are not delivered in the case of extrasystoles because they do not satisfy the criteria for a valid QRS complex. Even if they did, the RR interval for extrasystoles would not satisfy the condition for normal heart rate. Moreover, EP pulses are correctly not delivered at normal QRS complexes following the extrasystoles, again due to their RR intervals.

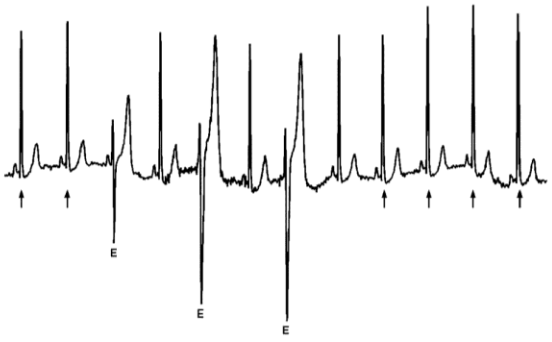

Figure 4. Delivery of EP pulses on ECG signal containing heart arrhythmias (ventricular extrasystoles). The arrows indicate the moments when EP pulses would be delivered. Absence of pulses at three extrasystoles (E) and at three normal QRS complexes following the three extrasystoles demonstrates the ability of the algorithm to prevent the EP pulses from being delivered in case of abnormalities in ECG shape or heart rate. The example belongs to the interval starting at 1:24:17 from the record s20101 of the LTST DB.

The presented algorithm is designed for robust operation in case of ventricular extrasystoles. However, the condition of no ventricular extrasystoles during the learning phase is desirable but not necessary. We tested how the algorithm would work if this condition was not fulfilled (data not shown). We selected four one-minute-long sections from the signal s30752 of the LTST DB which all included ventricular ectopy (phenomenon of seven or more singular ventricular extrasystoles per minute or any run of more than two ventricular extrasystoles). Careful examination of the performance of the developed algorithm on these four sections showed that presence of ventricular extrasystoles either within part I or part II of the learning phase does not affect the performance of the algorithm during the detection and decision-making phase, as long as their number is not very large (for example 10 or more extrasystoles in combination with 10 or fewer normal heartbeats).

5. Conclusion

The algorithm for online synchronization of electroporation (EP) pulse delivery with ECG presents a significant improvement over the existing practice of EP delivery with respect to the safety of the patient. This issue is becoming increasingly important because new applications of electrochemotherapy using endoscopic or surgical means to access internal tumours are being developed. Moreover, EP pulses of much longer durations used in some new applications of electroporation (such as electrogene therapy) would more likely coincide with the vulnerable period of the heart muscle if the pulse delivery were not synchronized with the heart activity. Therefore we developed an algorithm that allows EP pulses to be delivered only outside the vulnerable period of the heartbeat and prevents the pulses from being delivered in case of the appearance of some heart arrhythmias such as ventricular extrasystoles. The developed algorithm proved to be an effective tool for QRS detection and EP pulse delivery even in cases of numerous heart arrhythmias, which was confirmed by evaluation of the algorithm on ECG signals of the LTST DB database. The performance of the algorithm is significantly degraded only in presence of disturbances due to body movements that are similar to QRS complex, and in case of extrasystoles, which appear indistinguishable from normal heartbeats.

Implementation of the algorithm in instruments for clinical electroporation would essentially expand the applicability of electrochemotherapy due to a higher level of safety for the patient, as well as the suitability of this method for future applications in anatomical locations presently not yet accessible by existing electroporation devices and electrodes.

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PAPER VI

Antitumor effectiveness of electrochemotherapy: a systematic review and meta-analysis

B. Mali [a], T. Jarm [a], M. Snoj [b], G. Sersa [c], D. Miklavcic [a,*]

[a] *Department of Biomedical Engineering, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia*

[b] *Department of Surgery, Institute of Oncology Ljubljana, Ljubljana, Slovenia*

[c] *Department of Experimental Oncology, Institute of Oncology Ljubljana, Ljubljana, Slovenia*

[*] *Corresponding author. Department of Biomedical Engineering, Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 476 84 56; fax: +386 1 426 46 58. Email: damijan.miklavcic@fe.uni-lj.si.*

E-mail addresses:

barbara.mali@fe.uni-lj.si (B. Mali)

tomaz.jarm@fe.uni-lj.si (T. Jarm)

msnoj@onko-i.si (M. Snoj)

gsersa@onko-i.si (G. Sersa)

damijan.miklavcic@fe.uni-lj.si (D. Miklavcic)

ABSTRACT

Background

This systematic review has two purposes: to consolidate the current knowledge about clinical effectiveness of electrochemotherapy, a highly effective local therapy for cutaneous and subcutaneous tumors; and to investigate the differences in effectiveness of electrochemotherapy with respect to tumor type, chemotherapeutic drug, and route of drug administration.

Methods

All necessary steps for a systematic review were applied: formulation of research question, systematic search of literature, study selection and data extraction using independent screening process, assessment of risk of bias, and statistical data analysis using two-sided common statistical methods and meta-analysis. Studies were eligible for the review if they provided data about effectiveness of single-session electrochemotherapy of cutaneous or subcutaneous tumors in various treatment conditions.

Results

In total, 44 studies involving 1894 tumors were included in the review. Data analysis confirmed that electrochemotherapy had significantly ($p<.001$) higher effectiveness (by more than 50%) than bleomycin or cisplatin alone. The effectiveness was significantly higher for intratumoral than for intravenous administration of bleomycin ($p<.001$ for CR%, $p=.028$ for OR%). Bleomycin and cisplatin administered intratumorally resulted in equal effectiveness of electrochemotherapy. Electrochemotherapy was more effective in sarcoma than in melanoma or carcinoma tumors.

Conclusions

The results of this review shed new light on effectiveness of electrochemotherapy and can be used for prediction of tumor response to electrochemotherapy with respect to various treatment conditions and should be taken into account for further refinement of electrochemotherapy protocols.

Keywords:

electroporation; chemotherapy; bleomycin; cisplatin; treatment effectiveness; skin neoplasms

INTRODUCTION

Electrochemotherapy (ECT) is an antitumor therapy in which administration of a chemotherapeutic drug is followed by local application of electroporation pulses. Electroporation transiently permeabilizes tumor cell membranes, thus enabling diffusion of a chemotherapeutic drug (bleomycin or cisplatin) into the cells and increasing its cytotoxicity [1,2]. Other secondary mechanisms of ECT were also recognized [3–7]. Since the first clinical study in 1990 [8,9], ECT has been reported as highly effective, with complete response rates between 60-70% and objective response rates of about 80% [10–17], especially when the standard operating procedures (SOP) for ECT were followed [18]. ECT is routinely used in treatment of cutaneous and subcutaneous tumors due to high effectiveness, safety, limited toxicity, simplicity, cost-effectiveness, organ-sparing effect, and suitability for repetitive and neoadjuvant treatment [1,10,11,19–28]. New ECT approaches are currently being developed for treatment of deep seated tumors [29–33].

Effectiveness of ECT depends on extracellular drug concentration at the time of electroporation pulse delivery and on distribution of electric field inside tumor [34–36]. Other influential parameters related to patient, tumor and treatment characteristics (such as age; gender; tumor type, size and location; drug type, dose and route of administration; electrode type; protocol of electroporation pulse delivery) probably contribute to variability in tumor response to ECT, but their role has not been sufficiently explored [11,24,37].

The aim of this systematic review was to consolidate current experience with clinical ECT of cutaneous or subcutaneous tumors from the effectiveness point of view and to provide a transparent and objective framework for discussion on differences in effectiveness of clinical ECT. The main objectives were to evaluate: (a) overall effectiveness of ECT; (b) effectiveness of ECT in comparison to chemotherapeutic alone; (c) differences in effectiveness of ECT with respect to drug type and route of administration; (d) differences in effectiveness of ECT with respect to histological type of tumors.

MATERIALS AND METHODS

Search strategy

A systematic search of 16 bibliographic databases was performed to obtain articles regarding clinical ECT (Figure 1), using search terms “electrochemotherapy” and “clinical” and time range between 1st January 1991 and 18th October 2011. Language restriction to English was applied. Some references cited in these articles were screened to identify additional potentially eligible studies. Unpublished studies, abstracts, posters, reviews, editorials, lectures and commentaries were not included in this review.

Inclusion criteria for studies

Studies were included in systematic review if they provided:

(1) information about single-session ECT of cutaneous or subcutaneous tumors performed on human patients using bleomycin or cisplatin administered intratumorally or intravenously; and

(2) data for number of patients and tumors, tumor response (evaluated at least 4 weeks after treatment), chemotherapeutic drug, route of drug administration, and tumor type.

Studies were eligible for meta-analysis if they also provided:

(3) data for control tumors (i.e. tumors treated with chemotherapeutic drug only or electroporation pulses only, or no treatment); or

(4) data for at least two different histological types of tumors.

Study selection and data collection

Authors BM, TJ and GS independently examined studies identified with search strategy, performed selection of studies for further evaluation, read their full texts and extracted the relevant data (i.e. author and year of publication, number and gender of patients, number and type of tumors, tumor response, criteria for response assessment, chemotherapeutic drug, route of drug administration, duration of follow-up and the risk of bias). Disagreements between the authors were resolved by discussion. If the same data was reported in several studies, either the first published or the more comprehensive study was used. The authors of studies considered in this review were not contacted to provide additional data. The number of tumors included in the analysis was truncated to seven to prevent overestimation of effectiveness of ECT, when more than seven tumors with the same response were reported for the same patient (the approach adopted from Marty et al [11]).

Assessment of risk of bias

The risk of bias of included studies was assessed independently by 2 out of 3 authors (BM, TJ, GS) according to the recommendations of the Cochrane Collaboration [38]. Disagreements were discussed until consensus was reached. In addition to standard ratings (low, unclear or high risk of bias), a rating of "not applicable" was introduced for studies not including control tumors when judgment on sequence generation and allocation concealment was not possible. Reviewers were not blinded to the authors, location, funding and acknowledgements of the studies.

Outcome measures

The outcome measure of interest was the response of individual tumors to a single-session ECT (or control treatment). Tumor response in the evaluated studies was determined following WHO or RECIST criteria [39,40], or by biopsy or scanning. We classified the response of individual tumors as

complete response (CR), partial response (PR), no change (NC) or progressive disease (PD) according to the data reported in the studies. In addition, we introduced the objective response (OR; including CR and PR) and the no response (NR; including NC and PD) classifications. The complete and objective response rate (denoted as CR% and OR% respectively) were determined for each study.

Data analysis

Common statistical methods were used to analyze data from studies satisfying the first two criteria described above (Table 1A). The overall CR% and OR% (CR% and OR% columns in Table 2) were calculated separately from the pooled response data of individual tumors classified into various groups (either group of tumors treated with ECT or control tumors, or for various subgroups with respect to the chemotherapeutic drug, the route of drug administration, and the tumor type). Statistical comparison of CR% and OR% values between different (sub)groups was performed using Chi-square test. Differences were considered statistically significant for $p<.05$.

The overall CR% and OR% values result in a summary with equal contribution of all individual tumors. Consequently, the relative "weight" of each study in the overall results is proportional to its size. When applying analysis on data accumulated from studies performed by independent researchers, it is unlikely that the studies are functionally equivalent and of similar size. In such cases, a meta-analysis based on the random-effects model is the preferred method for pooling the data with the most reliable estimate of the summary effect [38,41]. However, only few studies were eligible for meta-analysis in this review; therefore, both approaches were used. Since there is no exact rule for the minimum number of studies to be included in a meta-analysis, we adopted the limit of six studies from some previous reviews [42–45].

A meta-analysis was used to evaluate the differences in antitumor effectiveness between ECT and chemotherapeutic drug alone and between different tumor types. The risk difference (RD) was used as the measure of the effect, defined as the probability of response in one group minus the probability of response in the other group. The between-study heterogeneity was assessed with the I^2 statistic. Small number of eligible studies prevented the use of funnel plots and subgroup analysis. The software was written in Matlab following published procedures [38,41].

A sensitivity analysis was performed to investigate the impact of studies with high risk of bias on the results of data analysis. In addition, the influence of the SOP for ECT on reported ECT effectiveness was evaluated by comparing the results of studies before and after year 2006 [18].

RESULTS

Search results

The initial search identified 1181 records after removal of duplicates. The study selection procedure is shown in Figure 1. Finally, 44 studies were appropriate for systematic review and data analysis (Table 1A). A much smaller subset of these studies was eligible for meta-analysis therefore the results of meta-analysis were treated as supplementary to the results of other statistical methods (Figure 1, Tables 1B and 1C).

Characteristics of the eligible studies

Characteristics of the studies used for systematic review are listed in Table 1A. In total, 413 patients and 1894 tumors were included. The studies were mostly non-randomized phase I or II studies and case reports. In only two studies tumors were randomized between different treatments but randomization was poorly conducted [49,56]. Studies eligible for meta-analysis comparing response of tumors to ECT with response of control tumors are listed in Table 1B. Among them, 13 studies were included for comparison of effectiveness between ECT and chemotherapy alone. Studies eligible for meta-analysis comparing response of different tumor types to ECT are listed in Table 1C. Among them, 8 studies were suitable for comparison of response to ECT between melanoma and non-melanoma tumors, and 6 studies for comparison of response to ECT between carcinoma and melanoma tumors. The results of risk of bias assessment of all studies are summarized in Figure 2.

Statistical analysis

ECT had significantly higher effectiveness than treatment with chemotherapeutic drug alone (Table 2). Namely, the overall CR% and OR% for ECT were 59.4% and 84.1% respectively and only 8.0% and 19.9% respectively for the chemotherapeutic drug alone. Treatment with electroporation pulses alone did not have any effect on tumor response. Similarly, the results of meta-analysis showed that ECT significantly increased the probability of CR% and OR% by 55% and 59% on average, respectively, in comparison to application of chemotherapeutic drug alone (Table 3).

A statistical comparison of response between different tumor types (melanoma, carcinoma and sarcoma) was performed separately for each chemotherapeutic drug and route of administration. No significant differences in overall CR% and OR% values were found between tumor types. Therefore the data for different tumor types was pooled for each chemotherapeutic drug and route of drug administration.

The overall CR% and OR% regardless of the drug and route of administration were 62.6% and 82.8% respectively (Table 2). However, effectiveness of ECT depended on the route of drug

administration with the overall CR% and OR% significantly higher for bleomycin administered intratumorally (72.7% and 85.8%, respectively) than intravenously (54.9% and 80.7%, respectively). There was no difference in effectiveness of ECT between bleomycin or cisplatin administered intratumorally.

A statistical comparison of response between different chemotherapeutic drugs and routes of drug administration was performed separately for each tumor type. No significant differences in overall CR% and OR% values were found between different drugs and routes of administration. Therefore the data for different chemotherapeutic drugs and routes of administration was pooled for each tumor type.

The overall CR% and OR% regardless of tumor type were 59.4% and 84.1% respectively (Table 2). However, effectiveness of ECT depended on the tumor types with the overall CR% and OR% significantly higher for non-melanoma (67.0% and 86.4%, respectively) than melanoma tumors (56.8% and 80.6%, respectively). Sarcoma tumors showed significantly better overall CR% and OR% than carcinoma tumors. Among different subtypes of carcinoma tumors, basal cell carcinoma tumors had significantly better response than melanoma tumors.

Results of meta-analysis comparing effectiveness of ECT for different tumor types showed significantly increased probability of CR and OR by 33% and 17%, respectively, for non-melanoma tumors in comparison to melanoma tumors ($p=.013$ for CR%, $p<.035$ for OR%) and significantly increased probability of CR% by 40% but insignificantly increased probability of OR% by 24% for carcinoma tumors in comparison to melanoma tumors ($p=.018$ for CR%, $p<.200$ for OR%; Table 3).

Sensitivity analysis

For the sensitivity analysis, six studies with an overall high risk of bias rating (Belehradek, 1993; Rudolf, 1995; Domenge, 1996; Rols, 2000; Curatolo, 2008, Campana, 2009) were removed from the statistical analysis [9,24,34,50,69,70]. All partial and overall responses to ECT were in general statistically insignificantly different from those reported in Table 2. Similarly, only minor changes in results were revealed for meta-analysis when comparing the response of tumors to ECT with response to chemotherapeutic drug only. Namely, increased probability of CR by 56% (CI of RD between 0.31 and 0.81) and of OR by 56% (CI of RD between 0.36 and 0.75) were obtained (compare to data in Table 3).

Additional sensitivity analysis was performed with respect to the year of study's publishing. When only studies published after publication of the SOP in 2006 were considered (25 studies, 1192 tumors), the overall CR% and OR% were 59.7% and 87.8% respectively (practically the same as CR% and OR% of 59.4% and 84.1% respectively in Table 2). When only studies published before 2006 were considered (19 studies, 592 tumors), the overall CR% and OR% were 61.1% and 77.4%, respectively.

When comparing CR% and OR% of studies published before and after the ESOPE study, the difference for OR% was significant but the difference for CR% was not (p<.001 for OR%, p=.565 for CR%).

DISCUSSION

Several clinical reviews have reported on effectiveness of ECT, but no systematic and comprehensive summary of effectiveness of clinical ECT has been published to date. In this systematic review, local effectiveness of a single-session ECT across all eligible studies was estimated as complete and objective response rate (denoted as CR% and OR% respectively) of 59.4% and 84.1% respectively (Table 2). The reviews of studies conducted before publication of the SOP for ECT reported similar values (CR% and OR% of 64% and 83%, respectively) [10,12], whereas later reviews reported only effectiveness of ECT for each study without appropriate synthesis of the data [17,19,20,26,79] with exception of two recent reviews summarizing effectiveness of ECT for melanoma and adenocarcinoma tumors [14,17]. We also separately determined the overall effectiveness of ECT for the studies conducted before (CR% and OR% of 61.1% and 77.4%, respectively) and after SOP publication (CR% and OR% of 59.7% and 87.8%, respectively). The OR% increased significantly after publication of the SOP, possibly as the result of adopting the SOP in the newer studies.

ECT has significantly higher effectiveness than chemotherapy alone (Table 2). Overall CR% and OR% of 8.0% and 19.9%, respectively, were achieved in control tumors treated with chemotherapeutic drug alone (bleomycin or cisplatin) applied at the same cumulative doses as in ECT (Table 2) without taking the intrinsic differences in effectiveness of bleomycin and cisplatin at used doses into account. Namely, bleomycin alone has a very low antitumor effect while cisplatin alone is moderately effective even without electroporation pulses [80–82]. From Table 2 it follows that ECT drastically improved the effectiveness of chemotherapy in general (CR% and OR% increased on average by around 55% and 63% respectively). Similar conclusions can be reached based on meta-analysis (Table 3). These results confirm that cytotoxicity of bleomycin and cisplatin is vastly increased when electroporation pulses are delivered to tumors in presence of sufficiently high extracellular concentration of chemotherapeutic drug [2,83]. The increase in effectiveness of ECT in comparison to chemotherapeutic drug alone in our study is higher than in study by Sersa et al [72] (increase or OR% by 40% for cisplatin), probably because we pooled the results for cisplatin and bleomycin together. The uptake of bleomycin by the cells is known to be more potentiated by electroporation pulses than the uptake of cisplatin [82].

However, in this review no significant difference between overall CR% or OR% was found between bleomycin or cisplatin administered intratumorally, which is also in agreement with the

ESOPE study results [11]. On the other hand, ECT with intratumoral administration of bleomycin or cisplatin revealed significantly higher overall CR% value than ECT with intravenous administration of bleomycin (Table 2). Advantages of intratumoral versus intravenous administration have been suggested in early studies [12], but no significant differences in effectiveness of ECT were found between intravenous and intratumoral administration of the drug in the ESOPE study [11]. Lower effectiveness of ECT with bleomycin given intravenously could be explained by insufficient volume coverage with the proper concentration of the drug in the tumor due to heterogeneous distribution of blood flow in tumors, or by insufficient interstitial drug concentration at the time of electroporation pulse delivery [4,11]. The cytotoxic activity of bleomycin and cisplatin is concentration- and time-dependent [81,84,85]. The SOP recommendations regarding the treatment window for application of electroporation pulses after administration of the drug were followed in most of the studies included in our review [18,34]. According to Front et al, the concentration of bleomycin in interstitial fluid around tumor is high enough for efficient ECT treatment for considerably longer period than suggested in the SOP for ECT [86]. Consequently, the insufficient interstitial drug concentration in the tumors due to improper timing of electroporation pulse delivery is an unlikely cause for lower effectiveness of ECT with intravenous bleomycin. On the other hand, the interstitial drug concentration in tumors cannot be predicted from the administered dose due to large variability in tumor drug uptake [86,87] and because of heterogeneous distribution of tumor blood flow within tumors, with the periphery usually being better perfused than the center [4]. Since the cytotoxicity of the drug depends on the extracellular concentration of the drug in the tumor, it is this parameter and not the administered dose of the drug that should be considered when planning effective ECT with intravenous bleomycin [86], for example by utilizing some noninvasive means for assessment of chemotherapy drug concentrations in tumor [88,89].

Significant differences in effectiveness of ECT between different tumor types were observed with melanoma tumors having in general lower CR% and OR% in comparison to all non-melanoma tumors combined, or carcinoma and sarcoma tumors separately (Tables 2 and 3). In general, sarcomas also responded significantly better than all carcinomas combined (Table 2). However, among all types of tumors, basal cell carcinomas (BCC) had the highest and squamous cell carcinomas (SCC) the lowest overall CR% and OR% (Table 2). Therefore the often repeated statement about equal clinical effectiveness of ECT regardless of tumor type appears to be unjustified. In some early studies in mice, different response rates to ECT were observed for different types of tumors with the best antitumor response being observed for fibrosarcomas [90,91] and the differences in intrinsic sensitivity of tumor cells to bleomycin were reported [91]. In some clinical studies, higher effectiveness of ECT was noticed in non-melanoma than melanoma tumors, but due to statistical insignificance the effectiveness of ECT was reported to be the same for all tumor types [11,17].

Differences in the response rate were pointed out by Mir et al, with BCC and SCC tumors having higher and lower response to ECT respectively than melanomas [68]. Additionally, the highest effectiveness of ECT in patients with BCC regardless of drug type and administration was reported in several other studies [13,53,55,59,60,71,92]. The difference in response between BCC and SCC tumors might be their different sizes; the SCC tumors were usually significantly bigger than BCC tumors and would therefore require repeated ECT treatments.

In meta-analysis of data from the eligible studies, some methodological factors were identified that are contributing to high heterogeneity (i.e. I^2 statistic) of included studies (Table 3), such as: differences in characteristics of patients; stage of the disease; size of tumors; protocols of electroporation pulse delivery; and inconsistent reporting of time of tumor response. Unfortunately, meta-analysis encompassing these confounding factors and thus estimating their influence on effectiveness of ECT is currently not possible because of too few studies eligible for meta-analysis published to-date.

CONCLUSIONS

The overall effectiveness of ECT in clinical setting and the differences in effectiveness of ECT of cutaneous and subcutaneous tumors due to heterogeneous treatment conditions (i.e. tumor type, drug type, route of drug administration) were systematically addressed for the first time. The identified differences could be used for a refined prediction of response to ECT of different tumor types, drug used and route of drug administration. This information should be taken into account for refinement and individualization of ECT treatment to further improve its effectiveness in cutaneous and subcutaneous tumors and to develop procedures for ECT of deep seated tumors.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest. Damijan Miklavcic holds patents of which some have been licensed to IGEA SpA; the producer of a clinical device used in some of the studies considered in this systematic review.

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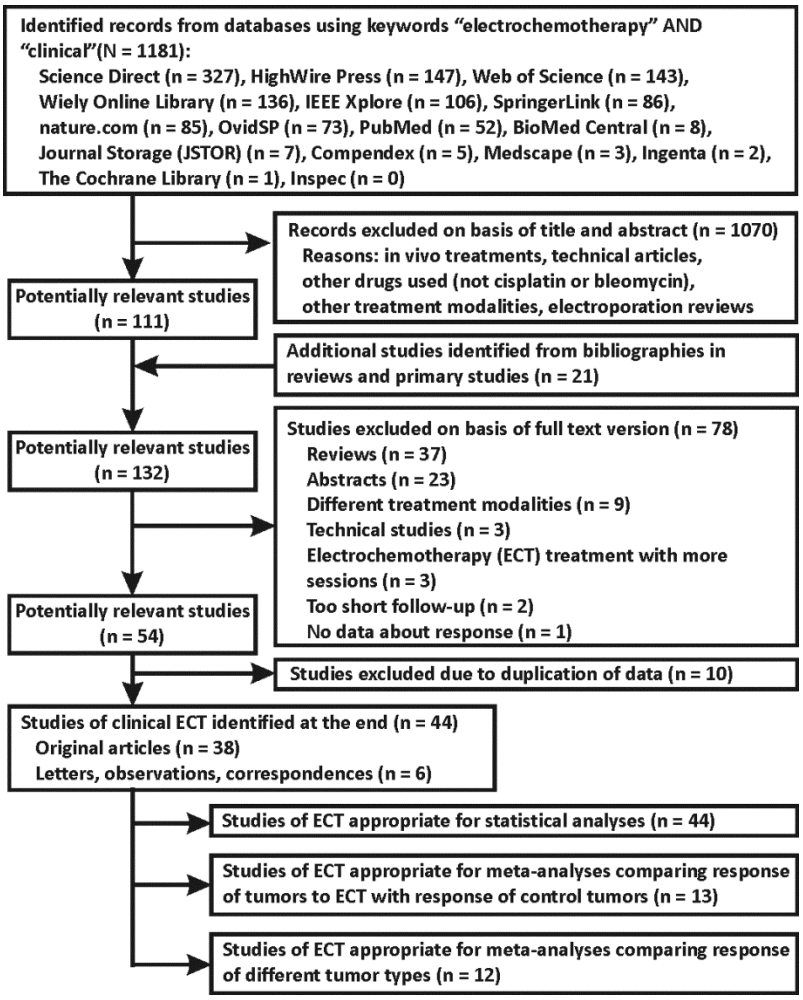

Figure 1: Selection process for the studies included in systematic review.

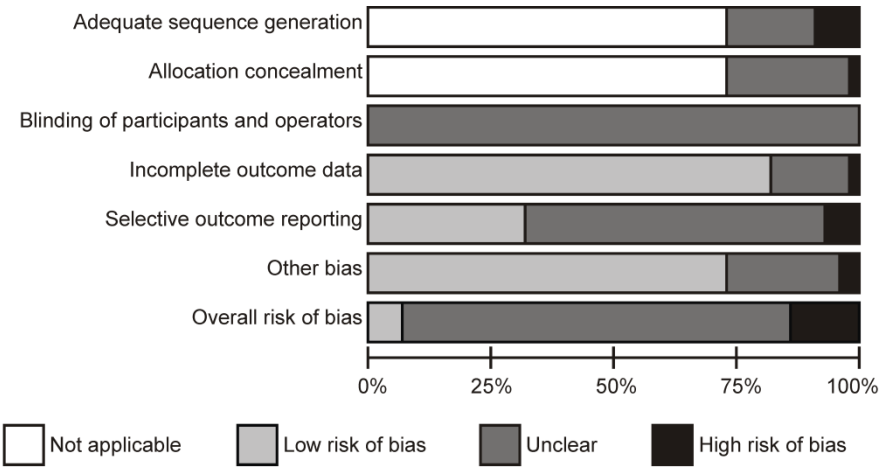

Figure 2: Assessment of risk of bias for the studies included in systematic review.

Table 1A: Summary of the studies and characteristics of tumors included in systematic review. Eligibility of the studies for meta-analysis is denoted in last two columns.

Original data			Data used in evaluation								Eligibility for meta-analysis	
First author, year published (reference)	No. of patients/ tumors	Gender of patients M/F	Included no. of patients/ tumors	No. of responses (%) CR (%)	PR (%)	NR (%)	Drug/ route	Type of tumor(s)	Response evaluation	Median follow-up in mo. (range)	Control tumors	Tumor types
Allegretti, 2001[a] [46]	14/14	9/5	3/3	3(100.0)	0(0.0)	0(0.0)	bleo i.t.	SCC	biopsy	31 (5.6–36.7)	no	no
Belehradek, 1993 [9]	8/42	8/0	5/26	23(88.5)	0(0.0)	3(11.5)	bleo i.v.	SCC	WHO	1.6 (1.0–8.3)	yes	no
Bloom, 2005 [47]	54/69	42/12	54/69	17(24.6)[b]	22(31.9)	30(43.5)	bleo i.t.	SCC	WHO, biopsy	>1 (-)	yes	no
Burian, 2003 [48]	12/12	11/1	12/12	10(83.3)	2(16.7)	0(0.0)	bleo i.t.	SCC	biopsy	1 (-)	no	no
Byrne, 2005[a] [49]	19/63	11/8	15/53	33(62.3)[b]	4(7.5)	16(30.2)	bleo i.t.	melanoma	WHO, biopsy	6 (3–6)	yes	no
Campana, 2009[a] [24]	52/608	20/32	52/267	125(46.8)	126(47.2)	16(6.0)	bleo i.t. or i.v. or combined	melanoma, breast cancer, sarcoma, SCC, HN cancer	RECIST	1 (-)	no	yes
Curatolo, 2008 [50]	1/-	1/0	1/7[c]	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	Kaposi sarcoma	-	14 (-)	no	no
Curatolo, 2009 [51]	1/-	1/0	1/7[c]	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	Merkel cell carcinoma	biopsy	6 (-)	no	no
Curatolo, 2011[a] [52]	23/532	13/10	18/114[c]	80(70.2)	34(29.8)	0(0.0)	bleo i.v.	Kaposi sarcoma	RECIST	18 (2–50.4)	no	no
Domenge, 1996 [34]	7/53	5/2	6/30[c]	7(23.4)	4(13.3)	19(63.3)	bleo i.v.[d]	HN SCC, salivary or breast AC	WHO	- (1–2)	yes	yes
Fantini, 2008 [53]	1/-	1/0	1/7[c]	7(100.0)	0(0.0)	0(0.0)	bleo i.t. or i.v.	BCC with squamous differentiation	-, biopsy	3 (2–9)	no	no
Garbay, 2006 [54]	1/-	1/0	1/7[c]	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	Kaposi sarcoma	WHO, biopsy	28.7 (-)	yes	no
Gargiulo, 2010 [55]	15/15	-/-	15/15	12(80.0)	3(20.0)	0(0.0)	bleo i.v.	BCC, SCC, Bowen disease	WHO	13 (3–24)	no	yes
Gaudy, 2006 [56]	12/30	9/3	9/23	17(74.0)	3(13.0)	3(13.0)	bleo i.t.	melanoma	WHO	4.8 (2–6)	yes	no

Gehl, 2006 [57]	1/8	1/0	1/7	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	melanoma	WHO	6 (-)	no	no
Gualdi, 2010 [58]	1/3	1/0	1/3	3(100.0)[e]	0(0.0)	0(0.0)	bleo i.t.	Kaposi sarcoma	biopsy	2 (-)	no	no
Heller, 1996 [59]	6/18	3/3	6/18	6(33.3)	7(38.9)	5(28.8)	bleo i.v.	melanoma, BCC, AC	WHO, biopsy	2.5 (2–5)	yes	yes
Heller, 1998 [60]	34/143	29/5	34/143	130(90.9)	12(8.4)	1(0.7)	bleo i.t.	melanoma, BCC, SCC, Kaposi sarcoma	WHO, biopsy	20 (7–28)	yes	yes
Kaehler, 2010 [61]	1/6	0/1	1/6	6(100.0)[f]	0(0.0)	0(0.0)	bleo i.t.	melanoma	biopsy	~4 (-)	no	no
Kis, 2011 [14]	9/158	2/7	9/158	37(23.4)	61(38.6)	60(38.0)	bleo i.v.	melanoma	WHO	7 (2–13)	no	no
Kubota, 1998 [62]	1/17	1/0	1/17	14(82.4)	3(17.6)	0(0.0)	bleo i.t.	transitional cell carcinoma	-	3 (-)	no	no
Kubota, 2005[a] [63]	1/8	0/1	1/8	7(87.5)	1(12.5)	0(0.0)	bleo i.t.	melanoma	biopsy	1.6 (-)	no	no
Landstrom, 2010 [13]	6/6	3/3	6/6	5(83.3)[g]	0(0.0)	1(16.7)[g]	bleo i.t.	HN BCC and SCC	biopsy	18.5 (3–24)	no	yes
Landstrom, 2011 [64]	5/5	3/2	5/5	5(100.0)	0(0.0)	0(0.0)	bleo i.t.	HN SCC, AC	biopsy	24 (24–24)	no	yes
Larkin, 2007 [65]	30/148	-/-	26/111	66(59.5)	24(21.6)	21(18.9)	bleo i.t. or i.v.	melanoma, AC, SCC, chondrosarcoma	WHO	- (2–12)	no	yes
Marenco, 2011[a] [66]	1/11	1/0	1/11	8(72.7)	0(0.0)	3(27.3)	bleo i.v.	SCC	-	2 (-)	no	yes
Marone, 2011 [67]	1/-	1/0	1/7§	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	metastatic eccrine porocarcinoma	-	5 (-)	no	no
Marty, 2006 [11]	61/290	20/41	41/171	126(73.7)	19(11.1)	26(15.2)	bleo i.t. or i.v. or cispl i.t.	melanoma, carcinoma, sarcoma	WHO	4.4 (2–12.7)	no	yes
Matthiessen, 2011 [15]	52/196	17/35	24/94	58(61.6)	18(19.2)	18(19.2)	bleo i.t. or i.v.	melanoma, BCC, SCC, AC, breast cancer	RECIST	- (2–6)	no	no
Mir, 1998[h] [68]	50/291	-/-	8/16[i]	3(18.7)	6(37.5)	7(43.8)	bleo i.v.	melanoma, HN SCC	WHO?	>1 (-)	no	no
Quaglino, 2008[a] [23]	14/233	8/6	14/233	136(58.4)	80(34.3)	17(7.3)	bleo i.v.	melanoma	WHO	21 (5–28)	no	no
Rebersek, 2004 [37]	6/12	1/5	6/12	4(33.3)	8(66.7)	0(0.0)	cispl i.t.	breast cancer	WHO	2 (2–6)	yes	no
Rols, 2000 [69]	5/61	2/3	5/61	6(9.8)	19(31.2)	36(59.0)	bleo i.v.	melanoma, HN SCC	WHO, scanning	1.6 (1–2)	yes	yes

Rudolf, 1995 [70]	2/24	1/1	2/24	22(91.7)	0(0.0)	2(8.3)	bleo i.v.	melanoma	WHO	4.1 (3.3–4.9)	yes	no
Sersa, 1998 [71]	4/19	3/1	1/4[j]	4(100.0)	0(0.0)	0(0.0)	cispl i.t.	BCC	WHO	>8 (-)	yes	yes
Sersa, 2000[k] [72]	10/82	2/8	10/82	66(80.5)	5(6.1)	11(13.4)	cispl i.t.	melanoma	WHO	>2 (-)	yes	no
Sersa, 2003[a] [73]	14/211	-/-	3/10	5(50.0)	2(20.0)	3(30.0)	cispl i.t.	melanoma	WHO?	2 (1.5–2.7)	no	no
Shimizu, 2003[a] [74]	1/1	0/1	1/1	0(0.0)	1(100.0)	0(0.0)	bleo i.t.	digital chondrosarcoma	biopsy	1 (-)	no	no
Snoj, 2005[a] [28]	1/1	1/0	1/1	0(0.0)	0(0.0)	1(100.0)	cispl i.t.	melanoma	-	1 (-)	no	no
Snoj, 2006 [75]	1/19	0/1	1/19	18(94.7)	0(0.0)	1(5.3)	cispl i.t.	melanoma	-	104 (3–104)	no	no
Snoj, 2007 [76]	1/244	0/1	1/7[c]	7(100.0)	0(0.0)	0(0.0)	bleo i.v.	melanoma	-	9 (-)	no	no
Snoj, 2009 [27]	1/1	1/0	1/1	0(0.0)	1(100.0)	0(0.0)	bleo i.v.	melanoma	-	4.9 (-)	no	no
Tijink, 2006 [77]	7/17	4/3	7/17	14(82.4)	3(17.6)	0(0.0)	bleo i.t.	SCC, melanoma, sarcoma, Merkel cell carcinoma	-, MRI, biopsy	12 (1–15)	no	yes
Whelan, 2006[a,l] [78]	1/1	0/1	1/1	0(0.0)	0(0.0)	1(100.0)	bleo i.t. and i.v.	breast AC	-	~2 (-)	no	no
Summary	**548/3672**	**237/202**	**413/1894**	**1125(59.4)**	**468(24.7)**	**301(15.9)**						

CR = complete response; PR = partial response; NR = no response; mo = month; - = no data; bleo = bleomycin; cispl = cisplatin; i.t. = intratumoral, i.v. = intravenous; SCC = squamous cell carcinoma; BCC = basal cell carcinoma; AC = adenocarcinoma; HN = head and neck.

[a] Only data for the first ECT session included.

[b] CR confirmed after 3 months by biopsy.

[c] The number of tumors per patient reduced to 7 if more than 7 tumors per patient with identical response reported.

[d] Bleomycin administered either i.v. or i.a.

[e] CR confirmed after 2 months by biopsy.

[f] CR confirmed after 1 month by biopsy.

[g] The responses confirmed after 2 months by biopsy.

[h] Not included in meta-analysis of tumor types because data is from different studies.

[i] Only 8 patients included because other data have been published in other articles (included are 5 patients with malignant melanoma tumors from the Ljubljana group and 3 patients with head and neck SCC tumors from the Reims group).

[j] Only 1 patient included because data for 2 patients with malignant melanoma tumors were published in Sersa et al, 2000, and 1 patient with BCC tumors was treated with more than one ECT sessions.

[k] The results from this article were updated thus having at least 2 months instead of 1 month follow up (data adopted from Sersa, 2006).

[l] A large nodule.

Table 1B: Summary of studies with any type of control tumors (chemotherapeutic drug only, electroporation pulses only or no treatment) included in meta-analysis comparing response of tumors to ECT with response of control tumors. For other details on these studies see Table 1A.

First author, year published (reference)	No. of patients/ tumors	Included no. of patients/ tumors	No. of responses (%)			Type of control
			CR (%)	PR (%)	NR (%)	
Belehradek, 1993 [9]	1/-	1/7[a]	0(0.0)	0(0.0)	7(100.0)	bleo i.v.
Bloom, 2005 [47]	8/37	8/37	0(0.0)	1(2.7)	36(97.3)	bleo i.t.
Byrne, 2005 [49]	15/19	15/19	5(26.3)	1(5.3)	13(68.4)	bleo i.t.
Domenge, 1996 [34]	2/-	2/7[a]	0(0.0)	0(0.0)	7(100.0)	bleo i.v.[b]
Garbay, 2006 [54]	1/-	1/7[a]	0(0.0)	0(0.0)	7(100.0)	bleo i.v.
Gaudy, 2006 [56]	9/15	9/15	2(13.3)	6(40.0)	7(46.7)	bleo i.t.
Heller, 1996 [59]	6/16	6/16	0(0.0)	0(0.0)	16(100.0)	bleo i.v.
Heller, 1998	3/6	3/6	0(0.0)	0(0.0)	6(100.0)	EP pulses
[60]	8/20	8/20	0(0.0)	1(5.0)	19(95.0)	bleo i.t.
Rebersek, 2004	6/8	6/8	0(0.0)	0(0.0)	8(100.0)	no treatment
[37]	6/6	6/6	0(0.0)	5(83.3)	1(16.7)	cispl i.t.
Rols, 2000 [69]	4/-	4/7[a]	0(0.0)	0(0.0)	7(100.0)	bleo i.v.
Rudolf, 1995 [70]	2/3	2/3	0(0.0)	0(0.0)	3(100.0)	bleo i.v.
Sersa, 1998	2/5	2/5	0(0.0)	0(0.0)	5(100.0)	no treatment
[71]	1/1	1/1	0(0.0)	0(0.0)	1(100.0)	EP pulses
	2/5	2/5	2(40.0)	2(40.0)	1(20.0)	cispl i.t.
Sersa, 2000	6/22	6/22	0(0.0)	0(0.0)	22(100.0)	no treatment
[72]	2/2	2/2	0(0.0)	0(0.0)	2(100.0)	EP pulses
	10/27	10/27	5(18.5)	5(18.5)	17(63)	cispl i.t.

CR = complete response; PR = partial response; NR = no response; EP = electroporation; - = no data; bleo = bleomycin; cispl = cisplatin; i.t. = intratumoral, i.v. = intravenous.

[a] The number of tumors per patient reduced to 7 if more than 7 tumors per patient with identical response reported.

[b] Bleomycin administered either intravenously or intraarterially.

Table 1C: Summary of studies included in meta-analysis comparing responses of tumors of different histological types. For other details on these studies see Table 1A.

First author, year published (reference)	No. of patients/ tumors	Included no. of patients/ tumors	No. of responses (%)			Type of tumor
			CR (%)	PR (%)	NR (%)	
Campana, 2009[a]	34/373	34/373	17(50.0)	15(44.1)	2(5.9)	melanoma
[24]	18/235	18/235	9(50.0)	9(50.0)	0(0.0)	non-melanoma (breast cancer, sarcoma, SCC, HN cancer)
Domenge, 1996	5/26	4/16	0(0.0)	4(25.0)	12(75.0)	HN SCC
[34]	1/20	1/7[b]	7(100.0)	0(0.0)	0(0.0)	salivary AC
	1/7	1/7	0(0.0)	0(0.0)	7(100.0)	breast AC
Gargiulo, 2010	9/9	9/9	7(77.8)	2(22.2)	0(0.0)	SCC
[55]	5/5	5/5	4(80.0)	1(20.0)	0(0.0)	BCC
	1/1	1/1	1(100.0)	0(0.0)	0(0.0)	Bowen disease
Heller, 1996	3/10	3/10	3(30.0)	2(20.0)	5(50.0)	melanoma
[59]	2/6	2/6	1(16.7)	5(83.3)	0(0.0)	BCC
	1/2	1/2	2(100.0)	0(0.0)	0(0.0)	AC
Heller, 1998	12/84	12/84	75(89.3)	8(9.5)	1(1.2)	melanoma
[60]	20/54	20/54	51(94.4)	3(5.6)	0(0.0)	BCC
	1/4	1/4	4(100.0)	0(0.0)	0(0.0)	Kaposi sarcoma
	1/1	1/1	0(0.0)	1(100.0)	0(0.0)	SCC
Landstrom, 2010	3/3	3/3	2(66.7)	0(0.0)	1(33.3)	HN SCC
[13]	3/3	3/3	3(100.0)	0(0.0)	0(0.0)	HN BCC
Landstrom, 2011	4/4	4/4	4(100.0)	0(0.0)	0(0.0)	HN SCC
[64]	1/1	1/1	1(100.0)	0(0.0)	0(0.0)	AC
Larkin, 2007	19/103	17/100	63(63.0)	21(21.0)	16(16.0)	AC
[65]	4/36	2/2	0(0.0)	1(50.0)	1(50.0)	melanoma
	5/6	5/6	3(50.0)	1(16.7)	2(33.3)	SCC
	1/2	1/2	0(0.0)	1(50.0)	1(50.0)	cervical carcinoma
	1/1	1/1	0(0.0)	0(0.0)	1(100.0)	synovial chondrosarcoma
Marty, 2006	32/190	20/98	65(66.3)	14(14.3)	19(19.4)	melanoma
[11]	29/100	21/73	61(83.6)	5(6.8)	7(9.6)	carcinoma and sarcoma
Rols, 2000	4/55	4/55	1(1.8)	18(32.7)	36(65.5)	melanoma
[69]	1/6	1/6	5(83.3)	1(16.7)	0(0.0)	HN SCC
Sersa, 1998	2/13	2/13	13(100.0)	0(0.0)	0(0.0)	melanoma
[71]	1/4	1/4	4(100.0)	0(0.0)	0(0.0)	BCC
Tijink, 2006	4/12	4/12	10(83.3)	2(16.7)	0(0.0)	SCC
[77]	1/3	1/3	3(100.0)	0(0.0)	0(0.0)	Merkel cell carcinoma
	1/1	1/1	1(100.0)	0(0.0)	0(0.0)	sarcoma
	1/1	1/1	0(0.0)	1(100.0)	0(0.0)	melanoma

CR = complete response; PR = partial response; NR = no response; SCC = squamous cell carcinoma; BCC = basal cell carcinoma; AC = adenocarcinoma; HN = head and neck.

[a] Tumor responses per patients.

[b] The number of tumors per patient reduced to 7 if more than 7 tumors per patient with identical response reported.

Table 2: The overall complete response rate (CR%) and objective response rate (OR%) were calculated from response data of individual tumors pooling individual tumor data of all studies together. CR% and OR% were calculated separately for tumors that: (1) served as controls; (2) were treated with ECT using different types of drug and routes of administration; (3) were of different histological types. Note that the sum of the numbers of articles, patients and tumors for subgroups is not necessarily equal to the value reported for all types because some studies, patients and tumors are included in several subgroups and some are not included in subgroups due to inseparable data. The numbers and letters in superscript are used to identify pairs of values (CR% or OR%) with statistically significant difference between them. The numbers in superscript indicate statistically significant differences between different subgroups within each group of studies. The letters in superscript indicate statistically significant differences between responses of tumors receiving ECT and responses of tumors receiving chemotherapeutic drug only. The statistical significances of Chi-square tests are listed in footnote of this table.

	No. of studies	No. of patients	No. of nodules	Overall response	
				CR%	OR%
Studies with control groups combined with respect to type of control					
all types	13	74	220	6.4	15.9
all drugs	13	74	176	8.0[a]	19.9[3,e]
bleomycin i.v.	6	16	47	0[1,b]	0[4,5,f]
bleomycin i.t.	4	40	91	7.7[c]	17.6[4,6,7,g]
cisplatin i.t.	3	18	38	18.4[1,2,d]	50.0[5,6,8,h]
EP pulses	3	6	9	0	0[8]
no treatment	3	14	35	0[2]	0[3,7]
Studies with ECT groups combined with respect to type of drug and route of administration					
all types	40	392	1421	62.6[a]	82.8[e]
bleomycin i.v.	19	137	835	54.9[9,10,b]	80.7[11,f]
bleomycin i.t.	16	192	414	72.7[9,c]	85.8[11,g]
cisplatin i.t.	7	63	172	75.6[10,d]	85.5[h]
Studies with ECT groups combined with respect to tumor type					
all types	44	413	1894	59.4	84.1
melanoma	22	150	922	56.8[12,13,14,15,16]	80.6[27,28,29,30,31]
non-melanoma	29	239	663	67.0[12]	86.4[27]
carcinoma	22	175	434	62.7[13,17,18]	81.1[32,33]
SCC	15	109	188	49.5[19,20,21]	69.7[28,34,35,36,37]
BCC	6	32	79	88.6[14,19,22,23,24]	100.0[29,34,38]
AC	6	28	130	59.2[22,25,26]	81.5[35,38,39,40]
sarcoma	8	25	138	73.9[15,17,20,23,25]	99.3[30,32,36,39]
Kaposi sarcoma	5	22	135	74.8[16,18,21,24,26]	100.0[31,33,37,40]
All studies with ECT groups combined					
	44	413	1894	59.4	84.1

CR% = complete response rate, OR% = objective response rate; EP = electroporation; i.v. = intravenous; i.t. = intratumoral; SCC = squamous cell carcinoma; BCC = basal cell carcinoma; AC = adenocarcinoma; statistical significances of Chi-square test: [1,4,27] .002, [2,7] .008, [3] .004, [5,6,9,10,12,14-16,19-22,29-34,36-40,a-h] <.001, [8] .006, [11] .028, [13] .036, [17] .017, [18,23] .010, [24] .015, [25] .011, [26] .007, [28] .001, [35] .020.

Table 3: Summary of the results of meta-analysis comparing effectiveness of ECT with respect to control group and effectiveness of ECT between different tumor types.

Comparison	No. of studies	No. of patients	No. of nodules	CR			OR		
				RD (CI_{low},CI_{up})	p(RD)	I^2	RD (CI_{low},CI_{up})	p(RD)	I^2
ECT vs. tumor controls receiving drug only	13	155	730	0.55 (0.33,0.77)	<.001	97.66	0.59 (0.44,0.74)	<.001	91.27
ECT in melanoma vs. non-melanoma tumors	8	175	592	-0.33 (-0.58,-0.07)	.013	95.65	-0.17 (-0.33,-0.01)	.035	95.37
ECT in melanoma vs. carcinoma tumors	6	79	363	-0.40 (-0.73,-0.07)	.018	96.50	-0.24 (-0.60,0.12)	.200	97.18

CR = complete response; OR = objective response; RD = summary risk difference for studies included in meta-analysis; CI_{low} and CI_{up} = the lower and upper confidence interval of RD, respectively; p(RD) = statistical significance of RD; I^2 = between-study heterogeneity.

PAPER VII

research article

Tumor size and effectiveness of electrochemotherapy

Barbara Mali[1], Damijan Miklavcic[1], Luca G. Campana[2], Maja Cemazar[3], Gregor Sersa[3], Marko Snoj[3], Tomaz Jarm[1]

[1] *Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia*

[2] *Sarcoma and Melanoma Unit, Veneto Region Oncology Research Institute (IOV-IRCCS), Padova, Italy*

[3] *Institute of Oncology Ljubljana, Ljubljana, Slovenia*

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Correspondence to: Prof. Marko Snoj, MD, Institute of Oncology Ljubljana, Zaloska 2, 1000 Ljubljana, Slovenia. Phone: +386 1 5879 796; Fax: +386 1 5879 407; E-mail: msnoj@onko-i.si; Prof. Tomaz Jarm, PhD, Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, 1000 Ljubljana, Slovenia. Phone: +386 1 4768 820; Fax: +386 1 4264 658; E-mail: tomaz.jarm@fe.uni-lj.si

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ABSTRACT

Background. Electrochemotherapy (ECT) is an effective and safe method for local treatment of tumors. However, relatively large variability in effectiveness of ECT has been observed, which likely results from different treatment conditions and tumor characteristics. The aim of this study was to investigate the relationship between tumor size and effectiveness of a single-session ECT.

Materials and methods. A systematic search of various bibliographic databases was performed and nine studies eligible for this study were extracted. Different statistical methods including meta-analysis were applied to analyze the data.

Results. The results of analysis based on data from 1466 tumors of any histotype show significantly lower effectiveness of ECT on tumors with maximal diameter equal to or larger than 3 cm (CR% of 33.3%, OR% of 68.2%) in comparison to smaller tumors (CR% of 59.5%, OR% of 85.7%). The results of meta-analysis indicated that ECT performed on tumors smaller than 3 cm statistically significantly increases the probability of CR by 31.0% and OR by 24.9% on average in comparison to larger tumors. The analysis of raw data about the size and response of tumors showed statistically significant decrease in effectiveness of ECT progressively with increasing tumor diameter. The biggest drop in CR% was detected at tumor diameters as small as 2 cm.

Conclusions. The standard operating procedures for ECT should be reexamined and refined for the treatment of large tumors. We propose that future clinical trials should include accurate ECT treatment planning and/or multiple ECT cycles, besides a prolonged observation for tumor response evaluation.

Key words: electrochemotherapy; cutaneous tumors; effectiveness; tumor size; meta-analysis

INTRODUCTION

Treatment of cutaneous and subcutaneous tumors using electrochemotherapy (ECT) has gained its role in routine clinical practice. The reason for an increasing use of ECT in clinics arises from favorable treatment characteristics, which are high effectiveness, safety, simplicity, low toxicity, possible application in an out-patient setup and cost-effectiveness.[1–7] The standard operating procedures (SOP) for ECT using the Cliniporator device were prepared during the ESOPE project.[1,8] The aim of the SOP document was to define guidelines for safe and effective ECT of cutaneous and subcutaneous tumors. Different treatment procedures were proposed within the SOP with respect to the number, size (maximal diameter) and depth of tumors. The SOP document was developed based on the experience from the leading European cancer centers using ECT, and tested during the ESOPE project in which also tumors larger than 3 cm in diameter were treated but they were excluded from ECT treatment evaluation reported in ESOPE study.[1] Nowadays, some researchers apply ECT also for the treatment of tumors larger than 3 cm.[9-11] Although general recommendations for ECT procedures on large tumors are given in the SOP, it is unclear whether this recommendations are appropriate for tumors with diameters larger than 3 cm.

The purpose of this study was therefore to examine the relationship between tumor size and effectiveness of single-session ECT, based on merging the evidence from different studies and to address the issue of the SOP for large tumors.

MATERIALS AND METHODS

Study selection and data extraction

All steps for a systematic review from PRISMA guidelines were applied in this study.[12–14]

The publicly available literature was systematically searched to obtain relevant published articles about clinical evaluation of effectiveness of ECT on tumors of various sizes. The following 16 databases were searched: Web of Science, Science Direct, PubMed, Wiley Online Library, OvidSP, HighWire Press, IEEE Xplore, SpringerLink, nature.com, Compendex, BioMed Central, Ingenta, Inspec, Journal Storage, The Cochrane Library, and Medscape. The search terms “electrochemotherapy” and “clinical” were used and the time span between 1st January 1991 and 22nd November 2011 was considered. Author BM first examined the titles and abstracts of the studies identified with the search strategy to narrow the initial selection of studies and then made the final selection based on full text reading. Authors TJ and GS independently checked the preliminary selection of studies. Bibliographies of original articles, review articles and relevant books were also screened to identify other potentially eligible studies. Articles published electronically were included but abstracts, posters, reviews, editorials, lectures and commentaries were not included in systematic review. In

addition, the data collected at the Institute of Oncology Ljubljana (denoted as IO data from this point onwards) was also recognized as appropriate and was hence included in the analysis.

A study was considered eligible for meta-analysis if the following criteria were met:

(1) inclusion of data for single-session ECT of cutaneous or subcutaneous tumors of any histotype performed on human patients;

(2) inclusion of data about number of patients and tumors, size and response of tumors, histotype of tumor; electrode type, drug type and route of administration;

(3) response of tumors evaluated at least 4 weeks after ECT treatment according to WHO or RECIST criteria, or with diagnostic imaging or biopsy;[15,16]

(4) data about size and response of tumors was reported in such a way that separation of tumors into two groups was possible: tumors with maximal diameter smaller than 3 cm and tumors with maximal diameter equal to or larger than 3 cm.

The cutoff dimension of tumor size of 3 cm in the last (fourth) criterion was selected because the majority of studies included in data analysis reported data of tumor responses only for group of tumors smaller and equal to or larger than 3 cm without details that would allow using a different cutoff value. The custom cutoff value can be set only for two studies with full access to raw data (IO data and data from Campana *et al.*[9]).

The following data was extracted from eligible studies by two of the authors (BM and TJ) independently: author and year of publication, number of patients, number, size and response of tumors, tumor histotype, electrode type, chemotherapeutic drug and route of its administration, criteria for tumor response evaluation, duration of follow-up and assessment of risk of bias of the study. Differences in extracted data between both authors were discussed to find the source of disagreement and to reach a common final decision. If the same data was used in two or more studies, either the first published or the more comprehensive study was included in the analysis. Authors of three studies included in the analysis were contacted for additional data, which were not included in published articles, but were needed for this study.[9–11]

The risk of bias of the studies was assessed following the Cochrane Collaboration recommendations.[14] Ratings for each bias issue (low, high or unclear bias) were extracted independently by two authors (BM and TJ) who were not blinded to names of the authors or locations of the studies. Ratings of both authors were compared and dissimilarities were discussed until consensus was reached. Studies were further rated as having an overall low (all bias issues rated with low), high (any bias issue rated with high) or unclear risk of bias (no bias issue rated with high and any bias issue rated with unclear).

In this study, the tumor response to a single-ECT application was evaluated. The tumor response was classified as either complete response (CR), partial response (PR), no change (NC) or

progressive disease (PD), according to the response criteria adopted in the studies (WHO or RECIST), or the pathologic response, assessed by biopsy.[15,16] Although WHO and RECIST criteria are different in some respects, these criteria are essentially equivalent for the evaluation of tumor response on individual lesions (the per-tumor effectiveness), which is the level of response considered in this study. CR is defined as a disappearance of tumor, PR as a decrease of at least 50% in the products of the two largest perpendicular diameters of the tumor (corresponding to tumor area), PD as an increase of more than 25% of lesion area. In all other cases, a response is determined as NC. Tumor response was determined not earlier than 4 weeks post treatment by two observations not less than four weeks apart. Tumors with CR and PR responses were further combined in the so called objective response group (OR) and tumors with NC and PD responses were grouped in the no response group (NR).

Statistical analyses

The overall effectiveness of ECT was determined across all eligible studies by pooling the response data of individual tumors of all studies together. For this purpose, complete and objective response rate (denoted as CR% and OR% respectively) were calculated across all eligible studies. The same calculations were also performed separately for the group of tumors with maximal diameter smaller and larger than (or equal to) 3 cm. CR% and OR% results of these two groups were compared using two-sided Chi-square test and the difference was considered statistically significant for $p < 0.05$.

The CR% and OR% values result in a summary in which all individual tumors from all studies contribute equally. Consequently, the relative contribution of each study to these values is proportional to its relative size. When applying statistical analysis on data accumulated from a series of studies that had been performed by researchers operating independently, it would be unlikely that all the studies were functionally equivalent. In such cases, a meta-analysis based on the random-effects model is generally the preferred method for pooling the results of independent studies.[14,17] By applying meta-analysis we obtained the most reliable estimate of the difference in effectiveness of ECT correlated to tumor size. The software for meta-analysis calculations was written in Matlab following the procedures published in the literature.[14,17] The so-called risk difference (RD) was used as the measure of the effect because of dichotomous nature of tumors' response data. RD is defined as the probability of response (either CR or OR) in one group minus the probability of the same response in the other group. The between-study heterogeneity was assessed with the I^2 statistic. The summary effect of meta-analysis was combined using a so-called random-effects model. This model considers the within-study variance and the between-studies variance and as a consequence the confidence interval (CI) of the summary effect is wider than in case of the fixed-effects model (thus requiring a larger difference between the two groups in order to find this difference significant). But by using the random-effects model, the larger studies (with many tumors) are also less likely to

dominate the overall effect and smaller studies (with few tumors) are less likely to be trivialized than with the fixed-effects model.[14,17] The difference between the two groups of tumors was considered statistically significant for p <0.05.

A sensitivity analysis was applied to investigate the influence of studies of high risk of bias on the overall results of data analysis.

The raw data about the size (maximal diameter of tumor) and response for tumors from article by Campana *et al.* were used for examination of relationship between tumor size and response.[9] Spearman's rank correlation coefficient and its significance were used for determination of statistical dependence between these two parameters. The tumors were also grouped by their size into four groups with 1 cm step size with the last group including all tumors equal to and larger than 3 cm, *i.e.* <1 cm, 1–2 cm, 2–3 cm and >3 cm. The differences in proportion of CR, PR and NR were tested between neighbor groups using Chi-square test in order to find the range of tumor size with statistically significant decrease of CR and OR and increase of NR with respect to its neighbor group. The same statistical tests were also performed on IO data. Due to a full access to IO data, the statistical comparisons of additional parameters (tumor area, volume, histotype and location; drug type and route of administration; current, voltage and energy per area delivered on tumor; electrode type; median follow-up) were performed between the groups. Rank Sum test on ordinal data and Chi-square test on nominal data were applied using statistical toolbox in Matlab and the difference was considered statistically significant for p <0.05.

RESULTS

Study selection and data extraction

The flow chart of the selection process for the studies included in data analysis is given in Figure 1. The initial search of 16 databases resulted in 1081 records after removal of duplicates but finally only eight articles satisfied all criteria.[9-11,18–22] The IO data from a clinical database of ECT performed on cutaneous and subcutaneous tumors at Institute of Oncology Ljubljana also met all the selection criteria and was therefore included as the ninth study. All these studies were non-randomized phase I or II studies.

The risk of bias was assessed for individual studies included in the analysis (Table 1). No assessment of overall risk of bias was possible for IO data because most of this data has not been previously published. Note that some of these data (about 40% of patients and 25% of tumors) has been published previously in the ESOPE study.[1]

The characteristics of the studies used for systematic review are shown in Table 2. In total, 1466 tumors and 197 patients were included. There were 252 (17.2%) tumors with maximal diameter larger than or equal to 3 cm, and 1214 (82.8%) tumors smaller than 3 cm.

Statistical analyses

Overall CR% and OR% of 55.0% and 82.7% were determined respectively, across all included studies irrespective of tumor size (Table 2). The results show higher effectiveness of ECT on tumors with the largest diameter smaller than 3 cm (CR% and OR% of 59.5% and 85.7%, respectively) in comparison to tumors with the largest diameter equal to 3 cm or larger (CR% and OR% of 33.3% and 68.2%, respectively). The differences of CR% and OR% between these two groups of tumors were statistically significant, both with p <0.001. Consequently, the proportion of tumors with NR (combining the cases of NC and PD after a single application of ECT) was statistically significantly higher for the larger tumors' group in comparison to the smaller tumors' group (NR% of 31.8% and 17.3%, respectively, p <0.001).

Similarly, the results of meta-analysis demonstrated that ECT performed on tumors smaller than 3 cm increases the probability of CR and OR by 31.0% and 24.9% on average, respectively, in comparison to tumors equal to or larger than 3 cm (summary RD values, see Figure 2). The results of summary risk difference (RD) for CR and OR were statistically significant with significances of <0.001 and 0.002, respectively.

For the sensitivity analysis, two studies (Rols *et al.*, 2000 and Campana *et al.*, 2009) with an overall rating of high risk of bias were removed from the statistical analysis to see if overall results are affected by the inclusion of studies with high risk of bias.[9,22] These two studies accounted for 22.4% of all tumors included in this study. The differences in CR% and OR% between groups of tumors of different size both remained statistically significant with p <0.001 (CR% and OR% of 62.5% and 87.9% respectively for smaller tumors, CR% and OR% of 35.4% and 70.8% respectively for larger tumors). When compared to CR% and OR% values in Table 1, the change of these values was relatively small in comparison to the variability of results between different studies. Similarly small changes in results were also found for meta-analysis when studies with a high risk of bias were excluded. Namely, RD for CR of 0.34 (CI between 0.13 and 0.55) and RD for OR of 0.26 (CI between 0.05 and 0.46) were obtained (compare these values to data in both summary lines of Figure 2). Both results for CR and OR however remained statistically significant with $p = 0.002$ and $p = 0.015$, respectively.

The analysis of raw data for the size and response for tumors from the study by Campana *et al.* showed that the effectiveness of ECT, defined as CR%, was decreasing progressively with increasing maximal tumor diameter (Spearman's rho = 0.418, p <0.001) (Figure 3A). The statistically significant drop in CR% (but not significant drop in OR% and increase in NR%) was detected between group of tumors of size <1 cm and 1–2 cm ($p = 0.017$), as well as between group of tumors of size 1–2 cm and 2–3 cm ($p = 0.001$), where the most evident drop in CR% was detected.

Similar results were obtained for the IO data, in which also similar tendency of decrease in effectiveness of ECT (expressed as CR%) with increasing size of treated tumors was detected (Spearman's rho = 0.129, $p = 0.078$) (Figure 3B). The maximal drop in CR% was detected between group of tumors of size 1–2 cm and 2–3 cm, and was statistically significant with p = 0.041. Due to full access to IO data, we were able to investigate if there was some other parameter beside the tumor size (such as tumor histotype and location; drug type, dose and route of administration; current, voltage and energy per area delivered on tumor; electrode type; median follow-up) that could be correlated with the observed difference in tumor response between these two size groups of tumors. Based on statistical comparison, these two groups of tumors of size 1–2 cm and 2–3 cm proved to be imbalanced with respect to upper listed parameters; therefore, no other parameter that would correlate with the difference in tumor response between these two size groups of tumors could be found. Among them, significant imbalance in proportion of melanoma and non-melanoma tumors, and drug type and route of administration used was identified.

DISCUSSION

The main prerequisites for an effective ECT treatment are an adequate extracellular concentration of the chemotherapeutic drug in the entire tumor at the time of pulse delivery and the coverage of tumor volume with an electric field able to permeabilize the cell membrane and therefore to enable drug uptake.[23–26] Sufficiently high electric field in the tumor tissue can be assured by delivery of pulses of adequately high voltage and appropriate positioning of the electrodes. In addition, some other conditions or parameters could be relevant, such as patient and tumor (histotype, size and location) characteristics and treatment parameters (drug, dose and route of administration, electrode type, protocol and timing of pulse delivery). In this study, we investigated the correlation between tumor size and effectiveness of ECT. Individual tumor data were gathered from heterogeneous non-randomized studies with various levels of additional information available; therefore we were not able to assess the possible cause-effect relationship between other parameters and the treatment response.

Our results showed that ECT was less effective on tumors larger than 3 cm in comparison to tumors smaller than 3 cm (CR% and OR% of 59.5% and 85.7%, respectively, versus CR% and OR% of 33.3% and 68.2%, respectively, Table 2). On the other hand, the no response rate (NR%) had more than doubled on larger tumors when compared to NR% on smaller tumors (from 14.3% to 31.8%, Table 2). The results of meta-analysis confirmed these findings, by showing that the effectiveness of ECT on the smaller tumors was significantly higher than on the larger ones, when the size limit between smaller and larger tumors was set to 3 cm (Figure 2) regardless of large heterogeneity of the included studies (Table 2). All results remained statistically significant when studies with an

overall high risk of bias were excluded. The sensitivity analysis thus showed that the overall results and conclusions are not affected by the inclusion of studies with high risk of bias. Therefore, our results can be considered with a higher degree of certainty.

The trend of decreasing ECT effectiveness with the increasing tumor size was clearly demonstrated with the analysis of raw data derived from the paper by Campana *et al.* and confirmed by the analysis of unpublished data from Institute of Oncology Ljubljana (IO data) (see Figure 3).[9] The results of the analysis based on the data from these two independent sources (the only two available for more detailed analysis) revealed that proportion of CR% was statistically significantly decreased already for tumors with maximal diameter around 2 cm (see Figure 3).

When treating large tumors with ECT, the SOP document suggests the administration of bleomycin by the intravenous route and use of needle electrodes in order to cover the whole tumor with sufficiently high electric field.[8] Almost all studies included in our survey were conducted according to the SOP recommendations, except for the study by Byrne *et al.* in which only intratumorally administered bleomycin was used and the study by Rols *et al.* in which strictly plate electrodes were used (Table 2).[18,22] Both these studies predate the publication of the SOP. Even though the SOP recommendations were generally followed in the studies included in the analysis, a relatively low response rates (CR% or OR%) were obtained in ECT treatment of tumors larger than 3 cm.

The first possible explanation for decreased effectiveness of ECT in tumors larger than 3 cm that should be considered is inadequate concentration of chemotherapeutic drug reached in the target tumor due to improper timing of pulse delivery. In the analyzed studies, pulses were applied either around 2 minutes after intratumoral bleomycin or cisplatin administration (which is within 10 min after drug administration as recommended in SOP), or within the therapeutic window of 8-28 minutes after intravenous bleomycin administration.[8,23] The interval between intratumoral drug administration and pulse delivery is adequate according to study by Cemazar *et al*.[27] The "optimal" therapeutic window for intravenous bleomycin administration (originally proposed by Domenge *et al.*) was actually determined based on data from a single patient on whom the ECT was performed in two sessions.[23] But according to the study by Front *et al.*, the concentration of intravenously administered bleomycin in interstitial fluid around tumor is high enough for efficient ECT treatment for considerably longer period after the injection than the "optimal" therapeutic window recommended within the SOP.[28] Plasma concentration of bleomycin declines biexponentially with a mean distribution half-life of approximately 24-30 min and mean elimination half-life of 2-4 hours,[29–31] which means that bleomycin concentration within tumors declines relatively slowly in the first two hours after intravenous administration. Therefore, if the insufficient extracellular drug concentration

in tumors was indeed responsible for the demonstrated lower effectiveness of ECT on tumors larger than 3 cm, it is very unlikely that it happened due to missed optimal therapeutic window for application of pulses. Nevertheless, further studies are needed to re-examine the current SOP recommendations for "optimal" treatment window. A more appropriate definition of the "optimal" therapeutic window should probably take into account other factors such as histotype, size and anatomical location of tumors to be treated, in addition to the drug type and time and route of its administration.

The second very likely reason for reduced effectiveness of ECT in large tumors is the insufficient exposure of the tumor to the drug, due to heterogeneous distribution of blood flow. It was reported that the periphery of the tumor is considerably better perfused than the inner portion, thus suggesting that the concentration of the drug in the center of the tumor can be lower than in the periphery of the tumor.[32–34] In addition, large temporal and spatial heterogeneity in blood flow is typical for tumors.[35] Higher drug concentrations in the inner portion of large tumors could be achieved by an appropriate combination of both intratumoral and systemic (intravenous) administrations.

The third possible explanation for the lower effectiveness of ECT in large tumors might be the insufficient coverage of the entire tumor volume with sufficiently high electric field. To overcome this problem, an individualized treatment planning based on radiological imaging could be adopted to determine the appropriate voltages based on the size, geometry and electrical properties of the target region.[36–39] Another option to maximize the tumor response could be to perform ECT treatment with fixed-geometry electrodes and their multiple and overlapped insertions.

The timing of response evaluation after ECT treatment for tumors should also be taken into consideration when interpreting the results of clinical studies. In this study, we considered tumors whose response assessment was performed at least 4 weeks after ECT, according to the SOP document.[8] However, longer healing time can be expected for larger tumors and 4 weeks after ECT may be too soon for evaluation of the response to ECT in many if not all large tumors. A healing time for smaller tumors is expected to be between 4 and 8 weeks, whereas for larger tumors (larger than 1.5 cm) can be prolonged to up to 10 weeks.[8] In this study, it turned out that 7 out of 9 studies reported response of tumors at least 8 weeks after ECT treatment. The remaining two studies (Rols *et al.*[22] and IO data) reported response evaluated less than 8 weeks after treatment only for small portion of tumors. If tumors with the response evaluated earlier than 8 weeks after ECT are not included into analysis, the results remain practically identical and the conclusions of this study remain unchanged. Nevertheless, for more accurate assessment of correlation between tumor size and response, longer follow-up observations should probably be more appropriate, especially because in general the kinetics of response of various tumors after ECT is unknown.

In this study, we considered exclusively the effect of a single-session of ECT. However, several clinical studies reported that the result of ECT on large tumors can be improved with repetitive treatments.[9,11] Moreover, ECT retreatment is not only recommended to achieve a better response in case of larger tumors, but also for smaller tumors unresponsive to the first ECT treatment.

In addition to investigation of correlation between tumor size and response, we intended to evaluate the influence of other tumor and treatment parameters on effectiveness of ECT (tumor area, volume, histotype and location; drug, dose and route of administration; current, voltage and energy per area delivered on tumor; electrode type; median follow-up). Such multivariate data analysis was unfortunately not possible due to unavailability of the details concerning these parameters for individual tumors in the analyzed studies. With such data reported in future clinical reports or with initiated ad hoc study, the reliable estimation of the most important influential parameters on response of large tumors will become possible.

In conclusion, the response of large tumors to ECT treatment seems not to be as good as that reported in smaller tumors. Tumor size starts to play a significant role in the final treatment outcome for tumors as small as about 2 cm in diameter. Therefore, we suggest that the SOP should be refined to improve the effectiveness of ECT for larger tumors. The optimal way to treat larger tumors should include individualized treatment planning to determine the appropriate electrode geometry and voltages, or, alternatively, the application of fixed-geometry electrodes with their accurate repositioning in order to overlap the treated volumes. Moreover, bleomycin could be administered combining both the intravenous and the intratumoral routes to achieve sufficient extracellular concentration in the portion of the tumor. The possibility of repetitive treatments on large tumors (already introduced in the clinical practice in some centers[9,11]) should be explicitly suggested within the SOP document, including the recommended interval between ECT cycles. Finally, for an accurate assessment of the correlation between tumor size and response to ECT in larger tumors, a longer follow-up (at least 3 months) could be required.

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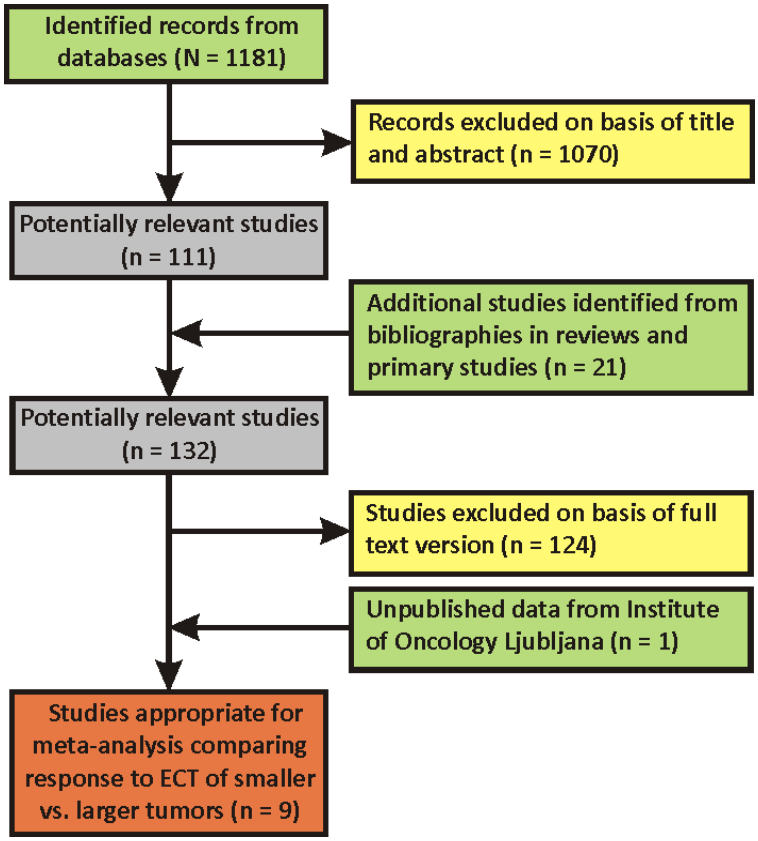
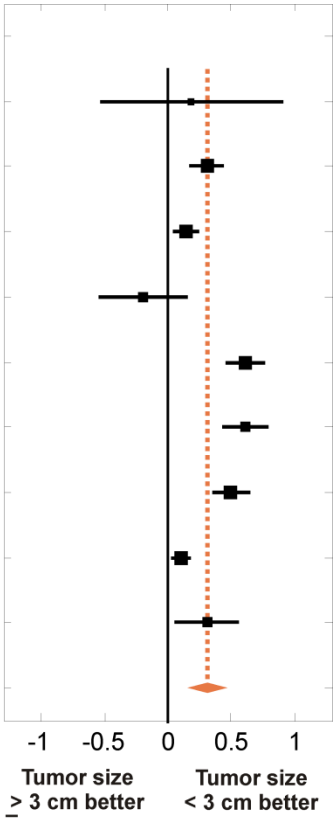


FIGURE 1. Selection process for the studies included in the data analysis.

(A) COMPLETE RESPONSE

Reference	RD	w%	CI low	CI up	N
Byrne, 2005	0.19	3.58	-0.54	0.92	18
Campana, 2009	0.31	12.98	0.18	0.44	267
Curatolo, 2011	0.14	13.51	0.04	0.24	489
Landstrom, 2010	-0.20	8.45	-0.55	0.15	6
Larkin, 2007	0.62	12.57	0.46	0.77	111
Matthiessen, 2011	0.61	11.98	0.43	0.80	94
Quaglino, 2008	0.50	12.65	0.35	0.66	233
Rols, 2000	0.10	13.82	0.03	0.18	61
IO data	0.31	10.45	0.05	0.57	187
Summary	0.31	100.00	0.15	0.47	1466

Test for overall effect: Z = 3,81 (p = 0.00014)
Heterogeneity: Q = 71.34, df = 8 (p < 0.0001), I^2 = 89%



(B) OBJECTIVE RESPONSE

Reference	RD	w%	CI low	CI up	N
Byrne, 2005	0.19	3.67	-0.54	0.92	18
Campana, 2009	0.26	15.50	0.13	0.40	267
Curatolo, 2011	0.05	16.85	-0.02	0.13	489
Landstrom, 2010	-0.20	9.35	-0.55	0.15	6
Larkin, 2007	0.53	13.14	0.31	0.75	111
Matthiessen, 2011	0.54	11.36	0.27	0.82	94
Quaglino, 2008	0.35	13.58	0.15	0.55	233
Rols, 2000	0.08	5.59	-0.47	0.63	61
IO data	0.25	10.95	-0.04	0.54	187
Summary	0.25	100.00	0.09	0.41	1466

Test for overall effect: Z = 3,11 (p = 0.00190)
Heterogeneity: Q = 37.41, df = 8 (p < 0.0001), I^2 = 79%

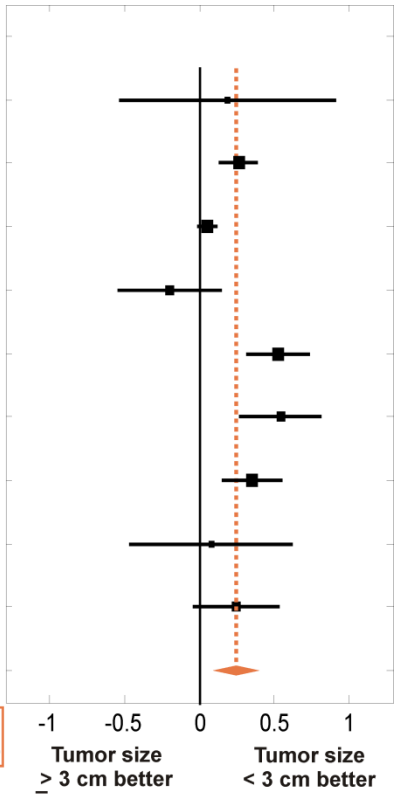


FIGURE 2. Results of meta-analysis. Data for individual studies and pooled results (Summary) demonstrating: (A) a statistically significant 30% increase in probability of CR for tumors smaller than 3 cm in comparison to tumors equal to or larger than 3 cm with ECT, and (B) a statistically significant 22% increase in probability of OR for tumors smaller than 3 cm in comparison to tumors equal to or larger than 3 cm with ECT.

RD = individual and summary risk difference for studies included in meta-analysis. w% = weight of study in comparison to all studies. CI low and CI up = the lower and upper confidence interval of RD, respectively. N = the number of tumors per each study and total number of tumors included in meta-analysis.

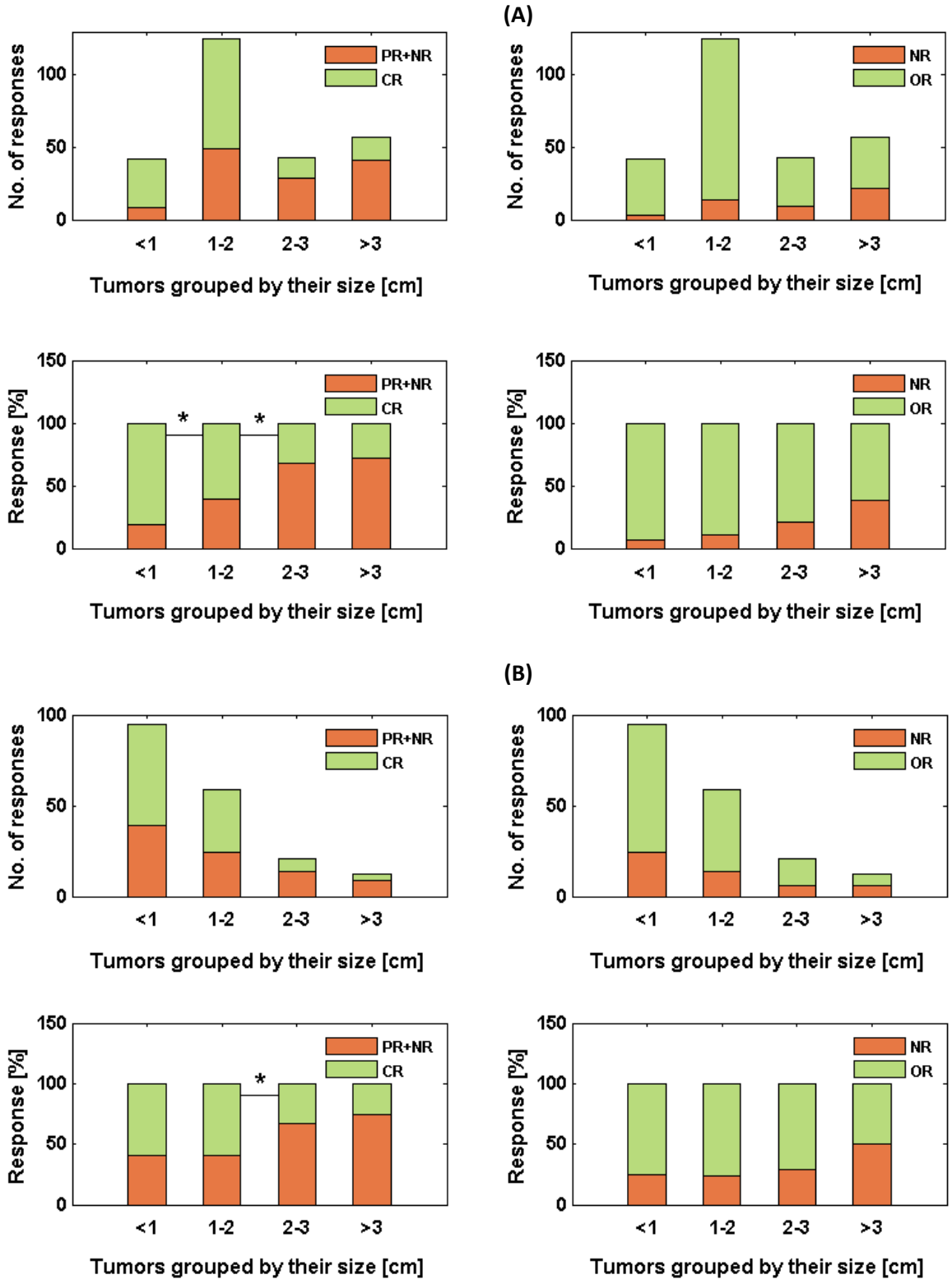


FIGURE 3. Number and proportion of tumor CR and OR to ECT with respect to tumor size for data: (A) from Campana et al and (B) from unpublished IO data. Tumors were grouped by their size using a 1 cm step. Each pair of neighbor groups, for which a statistically significant difference in proportion of CR and OR was found, is indicated with *. OR = objective response. CR = complete response. PR = partial response. NR = no response.

TABLE 1. Assessment of risk of bias for studies included in the analysis (except for IO data).

First author, year of publication, reference	Adequate sequence generation	Allocation concealment	Blinding of participants and operators	Incomplete outcome data	Selective outcome reporting	Other bias	Overall risk of bias
Byrne, 2005[18]	unclear	unclear	unclear	low	unclear	unclear	unclear
Campana, 2009[9]	unclear	unclear	unclear	unclear	high	high	high
Curatolo, 2011[10]	unclear	unclear	unclear	low	unclear	low	unclear
Landstrom, 2010[19]	unclear	unclear	unclear	low	low	low	unclear
Larkin, 2007[20]	unclear	unclear	unclear	low	unclear	unclear	unclear
Matthiessen, 2011[21]	unclear	unclear	unclear	low	unclear	low	unclear
Quaglino, 2008[11]	unclear	unclear	unclear	unclear	unclear	low	unclear
Rols, 2000[22]	high	unclear	unclear	low	high	unclear	high

TABLE 2. Summary of studies eligible for meta-analysis comparing the response to ECT of tumors smaller than 3 cm with tumors larger than 3 cm.

First author, year of publication, reference	No. of patients/tumors		No. of responses of tumors < 3 cm				No. of responses of tumors ≥ 3 cm				Drug, route	Electrode type, electroporator	Histotype of tumor(s)	Response evaluation	Median follow-up in mo. (range)
	All	Included	OR	CR	PR	NR	OR	CR	PR	NR					
Byrne, 2005[18]	19/63	15/18	11	11	0	5	1	1	0	1	bleo, i.t.	needle, Medpulser	melanoma	WHO, biopsy	6 (3-6)
Campana, 2009[9]	52/608	52/267	184	124	60	26	35	16	19	22	bleo, i.t. or i.v. or both	needle, Cliniporator	melanoma, breast cancer, sarcoma, SCC, head and neck cancer	RECIST	nd (2-21)
Curatolo, 2011[10]	23/532	23/489	330	225	105	38	102	57	45	19	bleo, i.v.	plate or needle, Cliniporator	Kaposi sarcoma	RECIST	18 (2-50.4)
Landstrom, 2010[19]	6/6	6/6	4	4	0	1	1	1	0	0	bleo, i.t.	needle, Medpulser	BCC and SCC	biopsy	18.5 (3-24)
Larkin, 2007[20]	30/148	26/111	82	64	18	8	8	2	6	13	bleo, i.t or i.v.	plate or needle, Cliniporator	melanoma, SCC, AC, chondrosarcoma	WHO	nd (2-12)
Matthiessen, 2011[21]	52/196	24/94	72	57	15	10	4	1	3	8	bleo, i.t or i.v.	plate or needle, Cliniporator	melanoma, SCC, AC, BCC, breast cancer	RECIST	nd (2-6)
Quaglino, 2008[11]	14/233	14/233	202	133	69	8	14	3	11	9	bleo. i.v.	plate or needle, Cliniporator	melanoma	WHO	21 (5-28)
Rols, 2000[22]	5/61	5/61	24	6	18	34	1	0	1	2	bleo, i.v.	plate, PS 15, Jouan	melanoma, SCC	WHO	1.6 (1-2)
IO data	52/379	32/187	131	98	33	44	6	3	3	6	bleo, i.t. or i.v., CDDP, i.t.	plate or needle, Cliniporator	melanoma, carcinoma, sarcoma	WHO	3.2 (1-16)
Summary (%)	**253/2226**	**197/1466**	**1040 (85.7)**	**722 (59.5)**	**318 (26.2)**	**174 (14.3)**	**172 (68.2)**	**84 (33.3)**	**88 (34.9)**	**80 (31.8)**					
Summary of all tumors (%)			**1212 (82.7)**	**806 (55.0)**	**406 (27.7)**	**254 (17.3)**									

OR = objective response (including CR and PR). CR = complete response. PR = partial response. NR = no response (including tumors with no change and progressive disease status). bleo = bleomycin. CDDP = cisplatin. i.t. = intratumoral route of administration. i.v. = intravenous route of administration. BCC = basal cell carcinoma. SCC = squamous cell carcinoma. AC = adenocarcinoma. mo. = month. nd = no data.

DECLARATION

The author herewith declares that the content of the thesis is a result of her own research work supervised by Assoc. Prof. Tomaž Jarm, Ph.D. The results which were obtained in collaboration with other colleagues are published in presented papers. Other assistance from colleagues is stated in the acknowledgments.

Barbara Mali