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Biological factors of the tumour response to electrochemotherapy: Review of the evidence and a research roadmap

Gregor Sersa [a, b, *], Katja Ursic [a, c], Maja Cemazar [a, d], Richard Heller [e], Masa Bosnjak [a, f], Luca G. Campana [g]

[a] Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska cesta 2, SI-1000, Ljubljana, Slovenia
[b] Faculty of Health Sciences, University of Ljubljana, Zdravstvena pot 5, SI-1000, Ljubljana, Slovenia
[c] Biotechnical Faculty, University of Ljubljana, Jamnikarjeva ulica 101, SI-1000, Ljubljana, Slovenia
[d] Faculty of Health Sciences, University of Primorska, Polje 42, SI-6310, Izola, Slovenia
[e] University of South Florida, 12901 Bruce B. Downs Blvd, FL-33612, Tampa, USA
[f] Faculty of Pharmacy, University of Ljubljana, Askerceva cesta 7, SI-1000, Ljubljana, Slovenia
[g] Department of Surgery, Colorectal and Peritoneal Oncology Centre, The Christie NHS Foundation Trust, Manchester, M20 4BX, UK

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ABSTRACT

The beneficial effects of electrochemotherapy (ECT) for superficial tumours and, more recently, deep-seated malignancies in terms of local control and quality of life are widely accepted. However, the variability in responses across histotypes needs to be explored. Currently, patient selection for ECT is based on clinical factors (tumour size, histotype, and exposure to previous oncological treatments), whereas there are no biomarkers to predict the response to treatment. In this field, two major areas of investigation can be identified, *i.e.,* tumour cell characteristics and the tumour microenvironment (vasculature, extracellular matrix, and immune infiltrate). For each of these areas, we describe the current knowledge and discuss how to foster further investigation. This review aims to provide a summary of the currently used guiding clinical factors and delineates a research roadmap for future studies to identify putative biomarkers of response to ECT. These biomarkers may allow researchers to improve ECT practice by customising treatment parameters, manipulating the tumour and its microenvironment, and exploring novel therapeutic combinations.

Development of electrochemotherapy

Electrochemotherapy (ECT) utilises short high-voltage electric pulses for enhanced local delivery of chemotherapeutic drugs, providing durable local tumour control. The procedure, which is simple in application and relatively inexpensive compared with other alternatives, involves an intratumoural injection of bleomycin (BLM) or cisplatin (CDDP) or an intravenous bolus infusion of BLM [1]. The application of electric pulses is achieved using dedicated electrodes of different sizes and geometries suitable for targeting small, large, superficial or deep-seated lesions. The treatment is currently applied predominantly as an alternative to surgical resection for the treatment of a wide range of superficial malignancies, mainly at European centres [2]. More recently, owing to the development of individually placeable electrodes, ECT has also been applied in the treatment of deep-seated neoplasms, including bone metastases, liver and soft-tissue neoplasms, and pancreatic cancer [2–4].

ECT standard operating procedures (SOPs) updated in 2018 codify the procedure into a simple algorithm that takes into account the number and size of tumours and the feasibility of local anaesthetics as the three guiding principles for selecting treatment parameters (anaesthetic technique, route of chemotherapy administration, and type of pulse applicator) [1].

Moreover, owing to consolidated experience and the growing range of indications, the procedure is continually being refined with a particular emphasis on low-demanding anaesthesiologic management [5] and de-escalation of chemotherapy doses [6]. Nowadays, ECT has been incorporated into several cancer treatment guidelines, including cutaneous melanoma, squamous cell

* Corresponding author. Department of Experimental Oncology, Institute of Oncology Ljubljana, Zaloska cesta 2, SI-1000, Ljubljana, Slovenia.
E-mail addresses: gsersa@onko-i.si (G. Sersa), kursic@onko-i.si (K. Ursic), mcemazar@onko-i.si (M. Cemazar), rheller@usf.edu (R. Heller), mbosnjak@onko-i.si (M. Bosnjak), luca.giovanni.campana@gmail.com (L.G. Campana).

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Abbreviations

BLM	bleomycin
CDDP	cisplatin
CR	complete response
CT	computed tomography
EAT	Ehrich ascites tumour
ECT	electrochemotherapy
GET	gene electrotransfer
Flt3l	Fms-like tyrosine kinase 3 ligand
HBV	hepatitis B
HPV	human papillomavirus
i-ECT	immune-Electro-Chemo-Therapy
InspECT	International Network for Sharing Practices of ECT
ISEBTT	International Society for Electroporation-Based Technologies and Treatments
MHC-1	major histocompatibility complex class 1
MRI	magnetic resonance imaging
OR	overall response
PDL-1	programmed death-ligand 1
PET/CT	positron emission tomography–computed tomography
SOP	standard operating procedure
TLR3	toll-like receptor 3
US	ultrasound

carcinoma, breast cancer, Merckel cell and basal cell carcinoma, soft tissue sarcomas and bone metastases guidelines [2,7,8].

Overall, the efficacy of ECT appears consistent across studies, particularly in the treatment of superficial malignancies, as demonstrated by the durable local control reported in most series [5]. Recently, a comprehensive analysis of the International Network for Sharing Practices of ECT (InspECT) database has provided evidence on the outcomes of 2482 tumours in 987 patients treated with ECT. After a single application, the most sustained response was observed in patients with basal cell carcinoma (*per-tumour* overall response [OR] and complete response [CR] rate, 96% and 85%, respectively) and Kaposi's sarcoma (98%/91%). In contrast, relatively lower rates were reported for patients with melanoma (82%/64%), breast cancer (77%/62%) and squamous cell carcinoma (80%/63%). In addition to histotype, previous irradiation and larger tumour size were negatively correlated with response to treatment [9]. These clinical factors are useful for guiding clinical decision making to select candidates, guide multidisciplinary team meetings, and council patients. Importantly, these factors are underpinned by a network of biological and physical factors affecting tumour response to ECT.

Since the procedure is standardized in terms of both equipment and electric pulse protocol, these largely unexplored biological factors are likely to explain the variation in response among patients. In addition to the biological factors, physical factors (*e.g.*, tissue electrical characteristics) also play roles in determining treatment outcomes [10,11]. However, these factors are beyond the scope of this review, and herein, we focus on biological factors.

The aim of this review is to summarise the current guiding clinical factors and delineate a research roadmap for future studies aimed at identifying putative biomarkers of response to ECT. These biomarkers may allow us to improve ECT practice by scustomising treatment parameters, manipulating the tumour and its microenvironment, and exploring novel combined approaches (Fig. 1).

Clinical factors associated with the response to ECT

Based on the clinical experience accumulated to date, the current clinical factors associated with tumour response to ECT are tumour histotype, tumour size and exposure to previous oncological treatments.

Tumour histotype

According to the recent comprehensive analysis of the InspECT register (n = 987 patients) different tumours have different degrees of sensitivity to ECT [9]. Among skin cancers, basal cell carcinoma seems to have the highest sensitivity, whereas melanoma is associated with relatively lower response rates. Differences in the response rate of tumours have been observed since the first clinical studies with ECT using either BLM [12,13] or CDDP [14] and also before the publication of the SOPs [1,15]. A more recent series indicated a CR rate of 66–100% for basal cell carcinoma, 55–80% for squamous cell carcinoma, and 25–55% for melanoma [5].

The systemic review and meta-analysis by Mali et al. [16] further highlighted the difference in the effectiveness of ECT in relation to tumour histotype. According to the analysis, ECT demonstrated higher activity in superficial sarcomas (OR 99.3%, CR 73.9%) compared with carcinomas (OR 81.1%, CR 62.7%) or melanoma (OR 80.6%, CR 56.8%). In contrast, the multi-institutional IMI-GIDO study by the Italian Melanoma Intergroup (n = 376 patients) did not detect any significant differences in response among histotypes in a multivariate analysis [17]. However, the issue concerning determinants of the tumor response is complex given the multiplicity of contributing factors (e.g., tumour size, anatomical location, and exposure to previous oncologic treatments), which may directly or indirectly influence the final effect. In the published series, patient and tumour heterogeneity make these evaluations challenging, and more comprehensive and consistent reporting is needed in future studies [18].

The current SOPs [1,15] do not recommend any procedural adjustment based on tumour histotypes. However, intrahistotype biologic heterogeneity can be more relevant than interhistotype differences [19]. Interestingly, this was observed in patients with superficially metastatic breast cancer treated with ECT. In the multicentre study by Cabula et al. [20], oestrogen receptor-positive and low Ki-67 index tumours, which are also referred to as the so-called *Luminal A-like* breast cancers according to the surrogate definition of intrinsic breast cancer subtypes [21], were associated with a significantly higher CR rate (74%) compared with the other subtypes (CR rate, 50–57%).

Therefore, in future studies, it is expected that ECT users will collect more detailed information regarding treated cancers, including not only histotype but also tumour subtypes. Importantly, their correlation with the response after ECT may allow to improve patient selection and customize treatment parameters.

Tumour size

The majority of ECT clinical studies have observed significant differences in tumour response according to the size of the treated lesions [22]. Recently, this observation was confirmed by a comprehensive analysis of the InspECT registry of 2,482 tumours [9]. Generally, it is accepted that lesions smaller than 3 cm in size, either superficial or deep-seated, exhibit a higher response rate than larger lesions [1,15,23]; however, the 3-cm cut-off is a matter

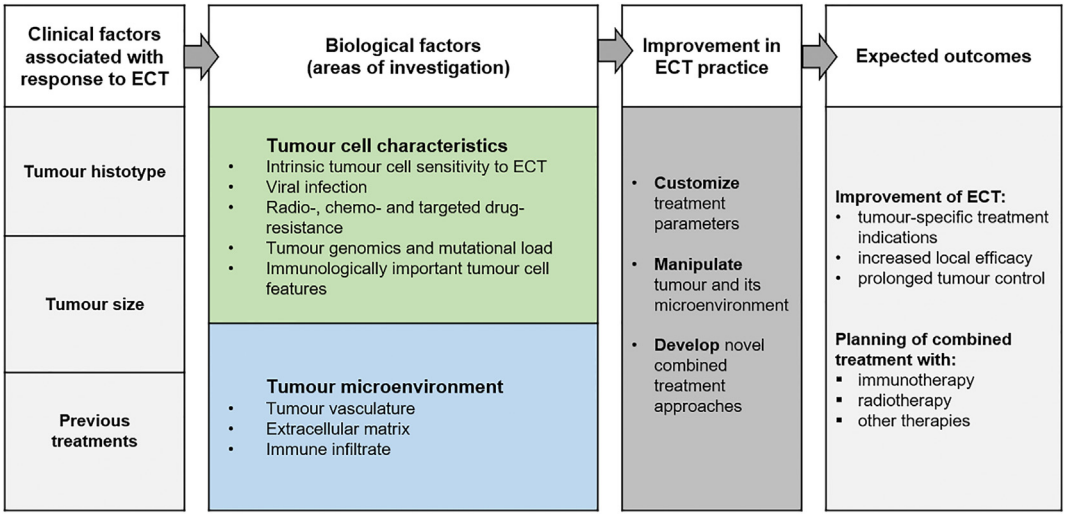

Fig. 1. Clinical factors associated with response to ECT and areas of biological factors investigation. Improved knowledge of ECT biological bases may allow customising procedure parameters and improving treatment efficacy.

of debate. For instance, Mali et al. [16] proposed a 2-cm cut-off, which was shown to be associated with the most significant reduction in the CR rate when analysing individual patient data. More recently, an analysis of the InspECT registry documented a steady progressive decrease in the CR response with the increase in tumour size, with rates consistently higher than 70% for tumours up to 1.5 cm in size [9]. Nonetheless, consistent response evaluation and further analyses are needed, possibly in more homogeneous populations to determine in each clinical context the most reliable cut-off to predict the response.

The explanations for the heterogeneity in tumour response may reside in the altered vasculature that occurs with tumour growth and the difference in cell susceptibility or aggressiveness in the hypoxic environment [24]. Additionally, coverage of large tumours with electric fields is challenging, and the probability of leaving a fraction of nonelectroporated cells is higher than it is for smaller lesions [25]. Thus, in the case of large and deep-seated tumours, the use of a treatment plan is recommended to achieve adequate electric field coverage of the target volume and permeabilization of cell membranes to chemotherapy [25].

Thus, the updated SOP [1] include specific recommendations for the treatment of large tumours: administration of BLM by the intravenous route, use of ultrasound (US) guidance to ensure accurate electrode placement, use of positron emission tomography–computed tomography (PET/CT) imaging to assess the response, and extended patient follow-up.

Previous treatments

The correlation between previous oncologic therapies and tumour response to ECT has been investigated in several studies. In a phase II study on patients with chest wall recurrence from breast cancer (n = 35), re-irradiation and different lines of systemic therapy did not hinder the response to ECT (CR rate, 54.3%) [26]. In contrast, recent evidence indicates that preirradiated tumours have a significantly lower response rate to ECT [27]. The trend towards a lower response rate in pretreated tumours was also observed in head and neck cancer patients previously treated with radiotherapy or chemotherapy [28,29].

Overall, it is acknowledged that ECT maintains sustained activity in tumours previously exposed to other therapies, hence its benefit in a palliative setting. Nonetheless, the elucidation of the underlying mechanisms of resistance may allow researchers to further improve its effectiveness and refine patient selection.

Areas of biological factors investigation

The clinical factors associated with tumour response to ECT are underpinned by the biological characteristics of the treated tumours. Herein, we discuss the two main areas of biological factors investigation (Fig. 1):

- Tumour cell characteristics
  - o Intrinsic tumour cell sensitivity to ECT
  - o Tumour viral infection
  - o Radio-, chemo- and targeted drug resistance
  - o Tumour genomics and mutational load
  - o Immunologically important tumour cell features
- Tumour microenvironment
  - o Tumour vasculature
  - o Extracellular matrix
  - o Immune infiltrate

Tumour cell characteristics

Intrinsic tumour cell sensitivity to ECT

Intrinsic tumour cell sensitivity to different therapeutic approaches is known to be of the utmost importance for therapeutic outcomes, and ECT is probably no exception. Early preclinical studies have indicated a variable response of tumours of different histotypes to ECT. One of our studies compared the responses of three types of tumours to ECT: SA-1 fibrosarcoma, B-16 melanoma and Ehrich ascites tumour (EAT) [30]. Subcutaneous lesions were treated by ECT using intravenous injection of BLM. Although the same conditions were used in the treatment of three tumour types, the SA-1 tumours were the best responders, with a 60% CR rate, whereas EAT and B16 melanoma exhibited CR rates of only 20 and 5%, respectively. The following study correlated the intrinsic sensitivity of tumour cells to ECT with the response rate. The difference in the response rate between SA-1 and EAT tumours *in vivo* correlated with the difference in intrinsic sensitivity of these two tumour cells either to BLM alone or after ECT [31]. Based on these studies, it was presumed that intrinsic sensitivity may predict

response to ECT. Moreover, the antitumour response to ECT also correlated with the tumour immune status from moderately immunogenic B16 and EAT to immunogenic SA-1 [30]. However, recent studies [32] noted that other biological factors, such as tumour vascularisation, may play an even more important role than intrinsic sensitivity in the determination of the antitumour response to ECT.

Tumour viral infection

Viral infection substantially changes tumour cell biology and elicits an immune response. The specific biology of human papillomavirus (HPV)-driven head and neck tumours results in an improved response to conventional therapies, including chemotherapy and radiation, as well as ECT [33,34]. Since head and neck cancer patients are frequent ECT candidates, information about the tumour response to ECT according to HPV status may be of interest. A preclinical study compared the response of parental and HPV-infected tumours to ECT with BLM and CDDP. Notably, both the HPV^+ and HPV^- tumours responded comparably to ECT with BLM, whereas the HPV^+ tumours were more sensitive to ECT with CDDP [33]. Viral infection may also be present in other malignancies treated with ECT, such as gynaecological cancers (HPV^+) and hepatitis B (HBV) positive hepatocellular carcinoma [35,36]. Therefore, in these cases, the correlation between HPV or HBV status and tumour response to ECT may be an interesting matter for investigation in future studies.

Radio-, chemo- and targeted drug resistance

ECT is predominantly used in refractory cancers after extensive application of other oncologic treatments. Therefore, these tumours have acquired varying degrees of radio- and chemoresistance and prove refractory. The influence of tumour cell acquired resistance to CDDP was explored during the early development of ECT [37]. Preclinical studies have indicated that CDDP-resistant cell lines and tumours had lower response rates to ECT than chemo-naïve tumours, where a significant response was also obtained [38]. Recently, an evaluation of the response to ECT was also performed in tumours with acquired radioresistance. ECT with BLM was equally effective on naïve and radioresistant tumours, whereas radioresistant tumours were more resistant to ECT with CDDP compared with radionaïve tumours [39]. All these outcomes were associated with increased DNA damage repair in resistant tumour cells [40].

Tumour genomics and mutational load

Genetic mutations are currently being exploited in the clinic as predictors for targeted therapy. For example, the *BRAF* mutation is a biomarker indicating the use of vemurafenib, dabrafenib and encorafenib in metastatic melanoma. The question of whether *BRAF* status might influence the response to ECT has been addressed only sporadically. *In vitro*, ECT was more effective in *BRAF*-mutated melanoma cells, and its effectiveness was potentiated in combination with vemurafenib [41]. In the clinic, the safety of ECT in combination with dabrafenib was described in a single patient with metastatic melanoma, in whom concurrent ECT with BLM during dabrafenib treatment ensured sustained local control without significant toxicity [42]. More research is needed to confirm the predictive value of *BRAF* and other current molecular markers in patients with metastatic melanoma and other cancers undergoing ECT.

Immunologically important tumour cell features

A number of studies have uncovered the powerful forecasting capability of tumour genomics [43], tumour mutational burden [44] and neoantigen burden [45] on the efficacy of cancer therapies, including immunotherapy. Moreover, other immunologically important features are being investigated in relation to the response to novel immunotherapy agents. For instance, in addition to programmed death-ligand 1 (PD-L1), which has been widely investigated as a biomarker, the expression of the major histocompatibility complex class 1 (MHC-1, HLA-1 in humans) may also be very important for the prediction of response [46]. However, there are no clinical data connecting tumour features related to the immune response and the response to ECT. Our recent murine study on ECT suggests that immunologically important tumour cell features, such as mutational burden and the expression of MHC-1, influence the response to ECT. Namely, ECT was more effective in more-immunogenic CT26 tumours compared with less-immunogenic 4T1 and B16F10 melanoma [47]. Furthermore, the study indicated that ECT increases MHC-1 and PD-L1 expression and thus favourably predisposes tumours to immunotherapies. The combination of ECT and immune checkpoint inhibitors is being explored in the clinic with promising preliminary results [48,49].

In addition to the mentioned immune markers, the mechanism and timeline of cell death after ECT are important. These parameters depend on the number of internalised drug molecules, as observed with BLM [50,51] and CDDP [52]. In addition to apoptosis, necrosis, mitotic cell death and caspase-independent necrotic-like cell death [53], and ECT with BLM [54], CDDP or OXA [55] also induces immunogenic cell death. Moreover, the release of damage-associated molecular patterns, such as adenosine triphosphate, calreticulin and nucleic acids, increases with increasing pulse amplitude in electroporation [56]. Whether different drugs used in ECT elicit a different degree of immunogenic cell death and other immunologically important events needs to be explored. We hypothesise that the local immunostimulatory signal *ie.* the type of cell death can vary depending on the drug type and dose. Therefore, different levels of immune stimulation with ECT may lead to different magnitudes of antitumour response.

The tumour microenvironment

In recent decades, the role of the tumour microenvironment in determining disease progression and treatment outcomes has become increasingly evident [51], but its role in response to ECT has never been consistently explored. Three major compartments can be identified that influence the response to ECT: the tumour vasculature, the extracellular matrix and the immune infiltrate.

Tumour vasculature

Tumour vasculature and perfusion are essential for drug delivery and drug distribution. It is well known that the tumour vasculature has an irregular architecture, and its organisation is chaotic compared to that of normal tissues. Moreover, the vasculature differs among histotypes, so vascularisation also predisposes the growth rate and drug delivery in different tumour types, especially when the route of drug administration is intravenous. Extensive work has been performed to explore the effect of the vasculature on the ECT tumour response and vice versa [32,57–59].

Recently [32], we compared the response to ECT between two animal models. The TS/A carcinoma model was more responsive to ECT with intravenous administration of BLM than the B16F1 melanoma model; however, melanoma cells *in vitro* were more susceptible to ECT than carcinoma cells. Then, we analysed the pharmacokinetics of the drug in both models and found better drug accumulation in the carcinoma model. Furthermore, the analysis of tumour vasculature showed that the carcinoma model had more-functional vasculature, as demonstrated by numerous but smaller vessels, than melanoma with less-functional vasculature, as indicated by fewer and larger vessels which are scarcely distributed

[32]. Thus, a better response to ECT was ascribed to better perfusion of the carcinoma, and tumour vasculature was proposed as a predictor of the response to ECT. Furthermore, no information is available on drug distribution after intratumoural injection, and the correlation between tumour size and the required injection volume remains unknown. We can presume that in smaller lesions, intratumoural injection provides uniform drug distribution, but there is no supporting evidence regarding larger lesions. The latest clinical data evaluation [27] also supports the presumption of differences in drug distributions in smaller and larger tumours after intatumoural ECT. Therefore, tumour vascularisation in relation to drug distribution deserves further investigation to acquire a consensus on its definition and to eventually assess its role as a putative biomarker. Importantly, the age-related variation of drug pharmacokinetics [60,61] should also be taken into account in future studies.

Another critical factor is the vascular disrupting action of ECT. In addition to tumour cells, ECT also targets endothelial cells, inducing apoptosis and thus producing a vascular disruption effect [62]. The data also indicate that electroporation more likely disrupts endothelial cells in small tumour vessels, whereas larger vessels seem to be preserved [59]. Thus, better tumour perfusion, *i.e.,* more functional vessels make a greater contribution to the vascular disrupting effect on the overall tumour response. For example, colorectal liver metastases are less vascularised than hepatocellular carcinoma and are less responsive to ECT [23,35]. Nonetheless, whether ECT using BLM or CDDP determines different intensities of the vascular disrupting effect remains an open question.

Extracellular matrix

The extracellular matrix, which is composed of noncellular connective tissue, is a critical component of the tumour microenvironment. The cross-talk between tumour cells and the extracellular matrix affects tumour growth, progression and metastasis [63]. Moreover, it has a crucial role in resistance to therapies. Presumably, in addition to the vasculature, a dense and aberrant extracellular matrix affects drug diffusion and pharmacokinetics, contributing to insufficient or heterogeneous drug distribution in solid tumours [64].

Currently, no data correlate ECT effectiveness and extracellular matrix characteristics. However, its structure can limit the access of therapeutic agents to their target through fibrosis, high interstitial pressure, and drug inactivation [65]. For instance, in gene electrotransfer (GET), plasmid transfection is hampered in tumours with high cell density and high content of collagen and proteoglycan [66]. Cell shape, size and density also affect tumour cell permeabilization and drug distribution and thus ECT effectiveness [66,67]. In addition to drug availability, the extracellular matrix affects the electric field distribution and conductivity [68,69]. Additionally, dense desmoplastic stroma can also interfere with the antitumour immune response, directly by acting as a physical barrier to immune cell infiltration or indirectly by establishing an immunosuppressive microenvironment [70].

Immune infiltrate

The immune system plays an essential role in tumour control as illustrated by the importance of immunoediting throughout cancer development (the three “E-s”, elimination, equilibrium and escape) [71]. For complete tumour elimination after therapies, an effective antitumour immune response is indispensable. This premise also relates to radiotherapy [72] and ECT. Two studies compared the tumour response to ECT in immunocompetent and immunodeficient mice. Namely, CRs were elicited in immunocompetent but not in immunodeficient mice [73,74].

In 1994, we assumed that the response to ECT with BLM correlated with tumour immune status. Namely, the highest response was observed in the most immunogenic tumours [30]. A similar result was observed when comparing more immunogenic SA-1 and less immunogenic TS/A tumours, with significant differences in the complete response rate [75]. Our recent study [47] also indicates a correlation between immune status and responsiveness to ECT. The more immunogenic tumours with higher immune infiltrate were more responsive to ECT. This finding probably correlates with the magnitude of induced immunogenic cell death [76] in different cancers.

These results indicate that the tumour response to ECT is dependent on both cancer cell characteristics and the properties of its microenvironment. As described in the next section, further investigation is needed to elucidate their impacts on the response to ECT.

Potential impact of biological factors on the improvement of ECT practice

Currently, decision-making in ECT practice is guided by clinical factors [27]. The investigation of the underlying biological factors may provide additional useful information and ultimately provide putative predictive biomarkers to test in future preclinical and translational studies.

If validated, these novel pieces of information can be implemented in ECT practice aiming to personalise treatment parameters, manipulate the tumour and its microenvironment and explore combined treatment approaches (Fig. 1). Furthermore, this information may provide more in-depth insight into how to improve treatment outcomes not only locally but also at the locoregional and distal levels (Fig. 2).

In this regard, the abscopal effect in association with ECT has been observed only sporadically, and only two reports have been published to date [77,78]. Nonetheless, preliminary findings support the investigation of ECT in combined strategies; *i.e., a* potentially promising approach is immune-Electro-Chemo-Therapy (i-ECT), which combines ECT and immunotherapies [48,79].

Tumour cell characteristics

Improved knowledge of tumour cell characteristics may open new avenues for refining the selection of ECT parameters.

Assessment of intrinsic tumour cell sensitivity, viral infection, radio-, chemo- or targeted drug-resistance, tumour genomics, mutational load and immunological features should be investigated wherever possible and correlated with the outcomes of ECT treatment. Moreover, the relationship between the type and dose of the chemotherapeutic drug used in ECT and the type of cell death it induces deserves investigation.

Selection of the chemotherapeutic drug, dosage, and route of administration in ECT can be adjusted based on tumour cell characteristics. If a specific drug is more effective for a specific tumour type due to 1) higher cytotoxicity, *i.e.,* electrochemosensitivity [67]; 2) better elicitation of the immune response, *i.e.,* type of cell death and modification of other immunological features [54,55,80,81]; 3) better drug distribution; or 4) better performance in combined treatment; then, we can expect better treatment results for a specific type of tumour (Fig. 2). For example, by knowing the molecular characteristics of resistant tumours, we can select an appropriate chemotherapeutic drug, which was demonstrated for HPV-infected tumours and tumours with acquired radioresistance [33,39].

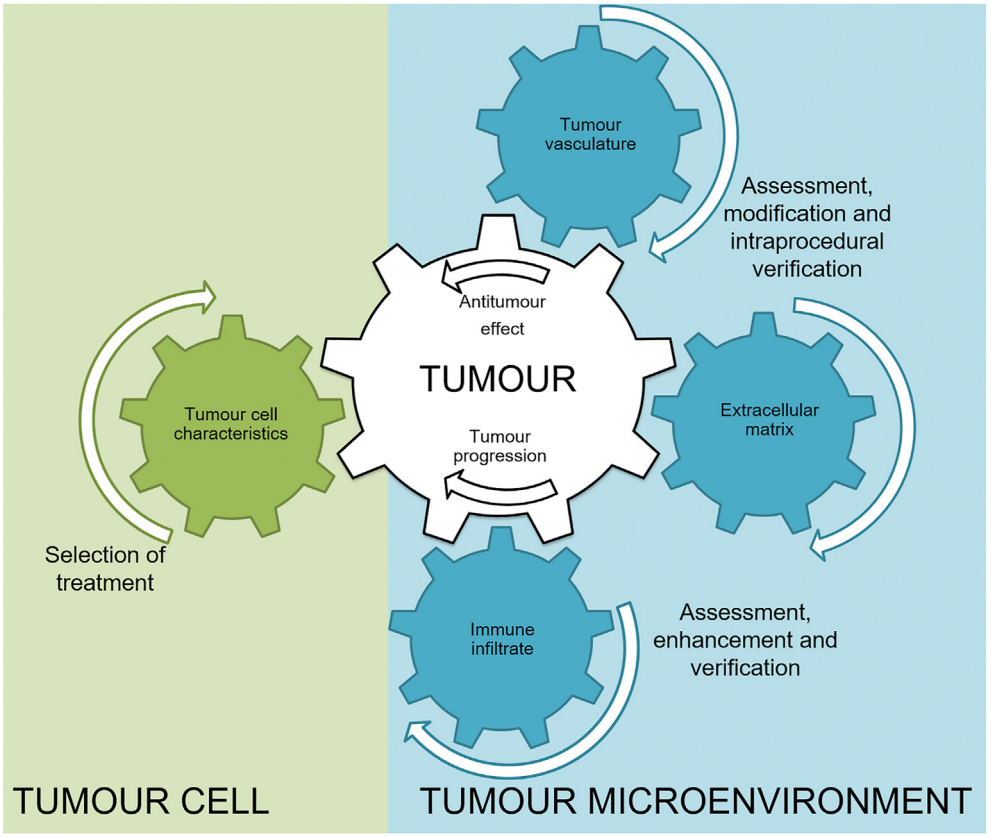

Fig. 2. Therapeutic implication of biological factors as putative biomarkers. The selection of treatment based on tumour cell characteristics and assessment, modification or enhancement of tumour microenvironment characteristics represent approaches to enhance the response to ECT.

Tumour microenvironment

Tumour vascularisation

Tumour vascularisation is a crucial area of investigation. Knowledge about the influence of the pattern and density of tumour vessels on the pharmacokinetics of specific drugs may correlate with the response to ECT and could be used to predict the response or to modify the microenvironment.

Assessment of tumour vasculature before ECT may forecast the antitumour response due to its influence on drug distribution and more pronounced vascular disrupting effect. The selection of chemotherapeutic drugs and treatment parameters used in ECT could impact 1) the vascular disrupting action of ECT [58,62] and 2) drug pharmacokinetics [32].

Modification of tumour perfusion could enhance drug delivery, distribution and consequently the effectiveness of ECT. For instance, mild hyperthermia, which is exploited in oncology practice to increase tumour perfusion and response to chemotherapy [82], can enhance the response of tumours that are less responsive to ECT. In addition, the combination of ECT with anti-angiogenic therapies may also be worth investigating [83]. Namely, the combination of anti-angiogenic therapies and radiotherapy is based on tumour blood vessel normalisation [84].

Intraprocedural verification of tumour perfusion with US can predict the adequacy of tumour coverage with an electric field as demonstrated in several studies on ECT [85,86] and irreversible electroporation [87]. The application of electric pulses induces a vascular lock that can be followed with sonography. In Fig. 3, tissue changes are visible in colorectal liver metastasis undergoing electroporation. The coverage of the whole tumour with an adequate electric field can be verified by US scan. This approach may also be useful in ECT applications to other large or difficult-to-approach malignancies [85,86].

Extracellular matrix

The extracellular matrix is another important feature of the tumour microenvironment. Namely, malignancies with larger cell sizes, lower cell densities and lower contents of non-cell-based components, such as proteins, glycoproteins and proteoglycans, respond better to therapies [63]. We envision that this information can be used in several ways:

The quantitative *assessment* of extracellular matrix contexture either histologically or by imaging may help to predict drug diffusion and consequently, cellular drug uptake during ECT procedure. Whether intravenous drug infusion is more suitable for dense/hard-consistency lesions, in which diffusion is hampered, and intratumoural application for softer tumours is not known. Similarly, how the injection volume in both types of administration routes impacts drug distribution should be explored. Therefore, depending on the characteristics of the extracellular matrix, the route of drug administration and injection volume could be adjusted.

Modification of the extracellular matrix may be another strategy to enhance the response to ECT [64]. In this regard, tumour mechanical properties can be modified to improve drug distribution [64]. For example, as observed with gene therapy using electroporation as a gene delivery system, hyaluronidase and/or collagenase pretreatment may enhance the distribution of molecules throughout the tumour or muscle [88–90]. In theory, this combinational strategy could be used to complement ECT when treating tumours with dense extracellular matrix. The drawback of this strategy may be that decrease in cellular adhesion could allow or

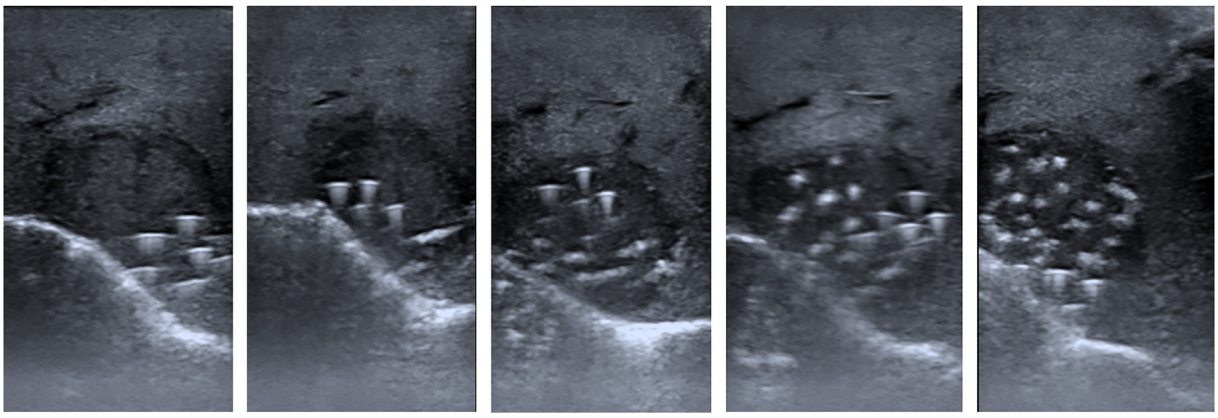

Fig. 3. Ultrasound-guided verification of tumour electroporation. The white spots represent the sites of hexagonal electrode insertion. After 5 applications of electric pulses, the whole tumour mass was covered and verified by following the vascular effects in the tumour, which is demonstrated by the hypoechoic tumour area.

even promote the metastatic process, this aspect must be explored as well [89,90]. Another approach could be targeting the transcription and cellular reprogramming of stromal cells by gene therapy [91] is the second strategy, currently being investigated preclinically, that could be combined with ECT.

Intraprocedual verification with noninvasive or minimally invasive imaging techniques is the most commonly used method to monitor the remodelling of the extracellular matrix. Currently, US, computed tomography (CT) and magnetic resonance imaging (MRI) are used in preclinical investigations and employed in clinical trials [92].

Tumour immune status

A better understanding of the pre- and post-ECT tumour immune status may also contribute improving the overall antitumour effect of ECT. Preclinically, ECT induces a local immune response and can be considered a type of *in situ* vaccination [48,79]. However, in clinical practice, the actual response may also be influenced by other factors, such as the fitness of the immune system and ongoing therapies. One of the ultimate goals is to exploit the local immune response to extend ECT effect from local to locoregional and, possibly, systemic.

Assessment of baseline tumour immunogenicity [93] and the characteristics of the local immune infiltrate [94,95] are potentially relevant information. Presumably, more immunogenic tumours exhibit a better response to ECT [75,47]. Furthermore, the induced immune response may vary upon the chemotherapeutic drug used and the treatment parameters, such as pulse parameters and the route of drug administration [56,96]. These parameters could impact elicitation of immunogenic cell death and the expression of immunologically important markers by *in situ* vaccination and thus suggest the timing of adjuvant immunotherapies.

The *enhancement* of the local and systemic immune response after ECT treatment could be achieved in three ways. First, ECT can be combined with *immune checkpoint inhibitors* [48]. A few clinical studies have demonstrated the proof of concept [49,97–99]. Patients undergoing anti-CTLA-4 or anti-PD-1 or anti-PD-L1 therapy also received ECT with no safety concerns. Moreover, preliminary data indicated that the treatment effect is potentiated locally. The second approach is combining ECT with general immunostimulators. For example, interleukin-12 is a potent cytokine that stimulates the innate and adaptive immune response and exhibits antiangiogenic effectiveness. However, multiple different cytokines and chemokines would be eligible for this type of treatment combination. Third, ECT could also be combined with toll-like receptor 3 (TLR3) agonist and Fms-like tyrosine kinase 3 ligand (Flt3l) or other molecules that recruit antigen-loaded and activated intratumoural cross-presenting dendritic cells [100]. Thus, the *in situ* vaccination effect of ECT can be improved with above mentioned immunotherapies (i-ECT), and the systemic immune response can be induced.

Assessment of the tumour's and patient's immune status before therapy and the induced antitumour immune response after ECT would help us to improve the ECT effect. However, no test has proven to be reliable to date. Several clinical studies have investigated the role of PET/CT imaging according to morphological (RECIST, iRECIST) and functional (PERCIST and PERCIT) criteria in the detection of antitumour immune responses or the prediction of clinical responses after immunotherapy. However, excluding invasive immunohistochemical evaluation, data on antitumour immune response assessment after ECT are not available.

Research roadmap

We envision that ECT efficacy can be improved by a better understanding of the underlying biological factors (Figs. 1 and 2). If validated, the introduction of biomarkers would ensure a better selection of treatment parameters as well as the development of novel combined treatment strategies (Fig. 4). Thus, these biomarkers would be instrumental for achieving the ultimate goal, that is, improvement of patient outcomes after ECT treatment.

This long-term goal can only be achieved through a joint collaboration between preclinical researchers and clinicians. Collaborative efforts are already in place with involvement of several research groups on the national and international level. The International network for sharing practices of ECT (InspECT; http://www.insp-ect.org/) and the International Society for Electroporation-Based Technologies and Treatments (ISEBTT; http://www.electroporation.net/) are examples. Since 2007, InspECT centres have established an ECT-dedicated registry to collect tumour, treatment, and patient outcome information, which includes data from 37 European centres. Furthermore, some of these groups have acquired consolidated experience in translational research on ECT [2]. To promote this effort, we propose a research roadmap for use by researchers and clinicians (Table 1).

In this scheme, it will be first of all necessary to explore the biological foundations of ECT further, through well-focused small-scale translational studies, to establish the bases for subsequent methodologically sound clinical investigations. These studies will focus on specific biological aspects (tumour cell characteristics,

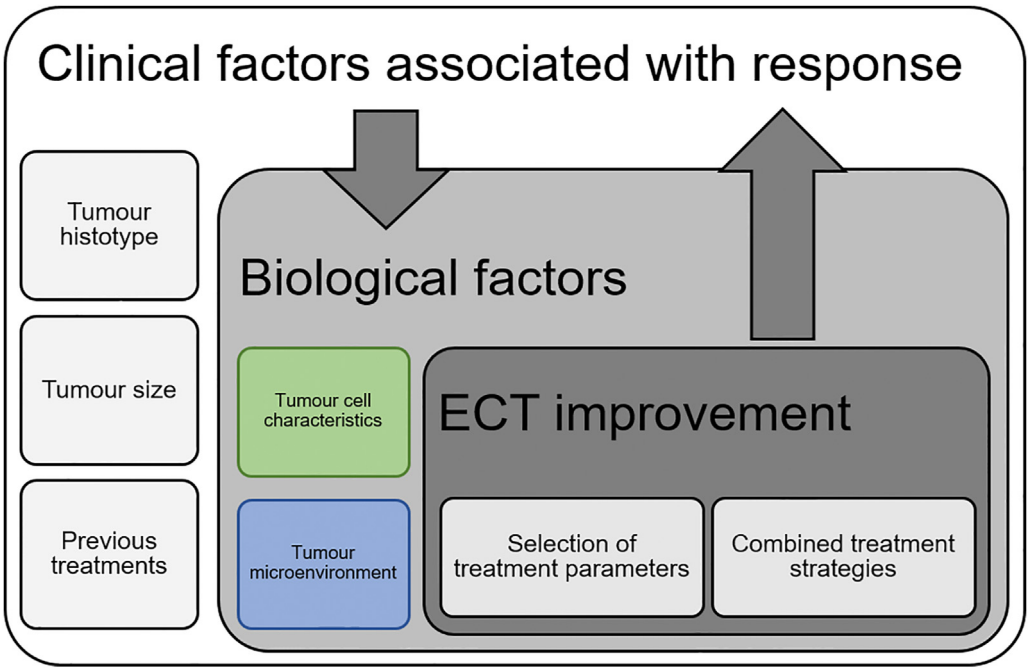

Fig. 4. In-depth knowledge of biological factors affecting the response to ECT may allow more precise selection of treatment parameters and development of novel combined treatment strategies.

Table 1
Candidate biological predictive factors to explain clinical predictive factors for the response to ECT. Suggestions for the roadmap to explore the biomarkers and their importance for the response to ECT at the preclinical level and suggestions for their translation into clinical practice for better effectiveness of ECT.

Areas of investigation	How to explore the biological factors preclinically	Requirements for translational activities	Clinical activities needed to individuate putative biomarkers
Tumour cell characteristics		**Clinical studies:**	
Intrinsic cell sensitivity to ECT	Determination in which tumours intrinsic sensitivity of tumour cells to chemotherapeutic drugs indicates tumour response to ECT.	• well-designed • focused • controlled	Verification if intrinsic tumour cell sensitivity indicates tumour response to ECT. Specifically, searching for inter- and intra-histotype variability in response.
Viral infection	Experimental evidence of viral infections on ECT effectiveness (HPV, HIV, Hepatitis, ect.)	• multi-institutional • quality of data	Correlative studies on ECT effectiveness according to viral infection.
Radio-, chemo- and targeted drug-resistance	Determination of radio-, chemo- and targerted drug-resitance on ECT response.	**Pre- and post-treatment tumour sample collection**	Correlative studies on cell and tumour characteristics in naïve and pretreated tumours or in tumours with genetic mutations (BRAF, HER^{+}, ect.).
Tumour genomics and mutational load	Correlation of mutational load and response to ECT.	**Biobank:** • biological factors • *ex vivo* predictive tests	Correlation of inter- or intra-histotype mutational burden and ECT response.
Immunologically important tumour cell features	Correlation of the immunologically important factors as well as cell death and the elicitation of the local and systemic immune response.		Analysis of patients' immune-response to ECT alone or in combined treatments according to immunologically important features and cell death.
Tumour microenvironment			
Tumour vasculature	Influence of pattern and vessel density on pharmacokinetics and tumour response.		Determination of tumour perfusion before treatment and correlateion to the ECT response and duration of the response.
Extracellular matrix	Drug access according to density of extracellular matrix.		Determination of extracellular matrix impact on ECT response and its duration.
Immune infiltrate	Characterisation of immune infiltrate pre- and post-ECT.		Determination of local and systemic immune response in patients before and after ECT.

tumour microenvironment) in a small number of patients to search for possible biomarkers. In this phase, the collection of blood and tissue samples in dedicated biobanks will be fundamental along with the integration of biological and clinical data.

Subsequently, the putative biomarkers will be investigated further on a larger scale through the conduction of well-designed clinical studies. Ideally, these studies should be supported by international scientific societies. Collaboration at the international level will also enable efficient patient recruitment and the collection of comprehensive, high-quality data, including quality of life, patient-reported outcomes, and extended follow-up information. At the same time, an improvement in the quality of ECT clinical studies and their reporting should be pursued through the dissemination of specific recommendations [18,101,102].

Therefore, we propose to unite the efforts fostering research on biological factors with the ultimate goal to improve patient outcome (Table 1).

Concluding remarks

Given the increasing recognition of ECT in cancer treatment, there is a need for a critical review on this therapy and a glimpse into the possible roadmap for future translational research.

ECT has made significant progress in the local treatment of several malignancies, ranging from primary skin cancer (basal cell and squamous cell carcinoma) to superficially metastatic tumours of various histotypes (breast cancer, melanoma, cutaneous sarcomas) and, more recently, deep-seated cancers (head and neck cancers and intra-abdominal malignancies).

However, studies on biological factors are lagging. Starting from the currently known clinical factors associated with the response to ECT, we have delineated a roadmap for future preclinical investigations and translational studies aimed at identifying putative predictive biomarkers of the tumour response. Importantly, this roadmap requires optimal coordination to maximise the collaborative efforts of preclinical and clinical research groups.

In conclusion, we propose to investigate the tumour and its microenvironment comprehensively in ECT patients. The individuation of putative biomarkers may allow improvements in treatment results, and if validated, these biomarkers may impact ECT clinical practice and ultimately improve patient outcomes. Additionally, a better understanding of the biological bases of ECT may help to develop rational combinations with other local and systemic therapies, including immunotherapy (i-ECT).

Author contributions

All authors contributed to the conception, drafting, and critical review of the manuscript, as well as provided final approval of the version to be submitted.

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CRediT authorship contribution statement

Gregor Sersa: Conceptualization, Writing – original draft, Manuscript review. **Katja Ursic:** Conceptualization, Data acquisition, Writing – original draft, Writing – review & editing, Manuscript review. **Maja Cemazar:** Writing – original draft, Writing – review & editing, Manuscript review. **Richard Heller:** Conceptualization, Writing – review & editing, Manuscript review. **Masa Bosnjak:** Data acquisition, Writing – review & editing, Manuscript review. **Luca G. Campana:** Conceptualization, Data acquisition, Writing – original draft, Writing – review & editing, Manuscript review.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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