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EDITORIAL



**DEAR READER, DEAR COLLEAGUES, DEAR
HYPERTHERMIA EXPERTS,**

You are reading the 39th volume of our Oncothermia Journal, featuring articles on modulated electrohyperthermia for 2024/2025. We apologize for the delay.

The first article discusses trends and future expectations in oncological hyperthermia, highlighting the promising prospects of immunogenic processes in mEHT applications. In technical terms, the computer simulation and experimental data show the heating dynamics of mEHT; the second article (Muratoglu et al.) demonstrates the appropriate role of the thermal component in the complex mEHT processes. Another technical article (Szucs et al.) on pulsed and modulated RF signals, based on recent research, shows potential to enhance ease of use and application versatility and to improve clinical outcomes. The core of mEHT lies in the synergy between thermal and nonthermal effects, as thoroughly discussed by Minnaar et al.

In vitro studies show the anticancer effects of mEHT against lymphoma cells (Schar et al.), supported by other preclinical and clinical reports. An interesting and stimulating study provides evidence of a naturopathic combination with mEHT, showing significant improvement in colorectal cancer patients (Izadi-Najafabadi et al.).

A real-world observational study indicates that individualized multimodal immunotherapy may extend overall survival while improving the patient's quality of life (Kampers et al.). An extended case report on residual glioblastoma shows the efficacy of the multimodal combination of therapies, including mEHT as the arch treatment (Chang et al.).

Important neoadjuvant observation shows the transformation of inoperable esophageal cancer to operable with mEHT application (Kuo et al.). The next article (Szasz et al.) deals with the biophysical basis of mEHT and its new theoretical and practical aspects.

A less-frequently studied field is the application of Mistletoe using mEHT. Kim et al.'s results suggest that the therapeutic application of Korean Mistletoe combined with mEHT is a potential candidate for cancer immunotherapy.

I believe this multitopic, informative material offers an excellent update on the successes of the mEHT method. You will find this issue of Oncothermia Journal useful for further development of the method and for use in your daily medical practice. Recent preclinical and clinical data highlight the superiority of mEHT, confirming its expected benefits and validating its role in oncology.

Dr. Andras Szasz

Professor, Chair, Biotechnics Department of Hungarian University of Agriculture and Life Sciences

**LIEBE LESER,
LIEBE KOLLEGEN,
LIEBE HYPERTHERMIE-EXPERTEN,**

Sie lesen den 39. Band unseres Oncothermia Journal, der Artikel über die modulierte Elektrohyperthermie (mEHT) für die Jahre 2024/2025 enthält. Wir bitten die Verzögerung zu entschuldigen.

Der erste Artikel behandelt Trends und Zukunftserwartungen in der onkologischen Hyperthermie und hebt die vielversprechenden Perspektiven immunogener Prozesse bei mEHT-Anwendungen hervor. Aus technischer Sicht zeigen Computersimulationen und experimentelle Daten die Dynamik der Erwärmung bei mEHT. Der zweite Artikel (Muratoglu et al.) verdeutlicht die angemessene Rolle der thermischen Komponente innerhalb der komplexen mEHT-Prozesse. Ein weiterer technischer Artikel (Szucs et al.) über gepulste und modulierte HF-Signale zeigt auf der Grundlage aktueller Forschung das Potenzial, die Benutzerfreundlichkeit und die Vielseitigkeit der Anwendung zu erhöhen sowie die klinischen Ergebnisse zu verbessern. Der Kern der mEHT liegt in der Synergie zwischen thermischen und nichtthermischen Effekten, wie von Minnaar et al. ausführlich erörtert wird.

In-vitro-Studien zeigen die krebshemmenden Wirkungen der mEHT auf Lymphomzellen (Schar et al.), die durch weitere präklinische und klinische Berichte gestützt werden. Eine interessante und anregende Studie liefert Hinweise auf eine naturheilkundliche Kombination mit mEHT, die bei Patienten mit kolorektalem Karzinom eine signifikante Verbesserung zeigt (Izadi-Najafabadi et al.).

Eine Beobachtungsstudie unter realen Versorgungsbedingungen deutet darauf hin, dass eine individualisierte multimodale Immuntherapie das Gesamtüberleben verlängern und gleichzeitig die Lebensqualität der Patienten verbessern kann (Kampers et al.). Ein ausführlicherer Fallbericht über ein residuelles Glioblastom zeigt die Wirksamkeit einer multimodalen Therapiekombination, bei der mEHT als zentrale Behandlung eingesetzt wird (Chang et al.).

Eine wichtige neoadjuvante Beobachtung zeigt, dass ein zunächst inoperables Ösophaguskarzinom durch die Anwendung von mEHT in einen operablen Zustand überführt werden kann (Kuo et al.). Der nächste Artikel (Szasz et al.) befasst sich mit den biophysikalischen Grundlagen der mEHT sowie ihren neuen theoretischen und praktischen Aspekten.

Ein seltener untersuchtes Gebiet ist die Anwendung von Mistelpräparaten in Kombination mit mEHT. Die Ergebnisse von Kim et al. legen nahe, dass die therapeutische Anwendung der koreanischen Mistel in Kombination mit mEHT ein potenzieller Ansatz für die Krebsimmuntherapie sein könnte.

Ich bin überzeugt, dass dieses thematisch vielfältige und informative Material einen ausgezeichneten Überblick über die Erfolge der mEHT-Methode bietet. Sie werden diese Ausgabe des Oncothermia Journal sowohl für die weitere Entwicklung der Methode als auch für ihre Anwendung in Ihrer täglichen medizinischen Praxis als hilfreich empfinden. Aktuelle präklinische und klinische Daten unterstreichen die Überlegenheit der mEHT, bestätigen die erwarteten Vorteile und bekräftigen ihre Rolle in der Onkologie.

RULES OF SUBMISSION

As the editorial team we are committed to a firm and coherent editorial line and the highest possible printing standards. But it is mainly you, the author, who makes sure that the *Oncothermia Journal* is an interesting and diversified magazine. We want to thank every one of you who supports us in exchanging professional views and experiences. To help you and to make it easier for both of us, we prepared the following rules and guidelines for abstract submission.

Als redaktionelles Team vertreten wir eine stringente Linie und versuchen, unserer Publikation den höchst möglichen Standard zu verleihen. Es sind aber hauptsächlich Sie als Autor, der dafür Sorge trägt, dass das *Oncothermia Journal* zu einem interessanten und abwechslungsreichen Magazin wird. Wir möchten allen danken, die uns im Austausch professioneller Betrachtungen und Erfahrungen unterstützen. Um beiden Seiten die Arbeit zu erleichtern, haben wir die folgenden Richtlinien für die Texterstellung entworfen.

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ONCOLOGICAL HYPERTHERMIA: WHERE TO GO FROM HERE?

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ABSTRACT

Oncological hyperthermia is one of the most versatile forms of oncotherapy. It can complement every conventional treatment method and be applied to any tumorous cancer, irrespective of its stages and localization. Numerous technical realizations are conventionally compared by their thermal effect, measured by temperature. However, nonthermal (mainly electric) excitation effects are more recognized nowadays. The technical variants alter the synergy between thermal and nonthermal energy components. Nonthermal energy absorption– induced molecular mechanisms include essential behaviors like selectivity and immunogenicity. The nonthermal electromagnetic effects excite molecular changes, intracellular signals, gene expressions, and many other chemical reactions. Their synergy with thermal conditions is based on the Arrhenius law, which describes the rapid growth of chemical reactions with temperature. A unique technical realization of hyperthermia, modulated electrohyperthermia (mEHT) tries to optimize the thermal and nonthermal effects. The results look very perspective, containing the high accuracy of targeting the tumor cells, the immunogenic cell death, and the activation of tumor-specific immune reactions restoring the healthy immune surveillance to destroy the cancer.

Keywords

Thermal, Nonthermal, Modulated Electrohyperthermia, Selection of Tumor Cells, Tumor-Specific Immune Activity, Abscopal Effect

1. INTRODUCTION

Hyperthermia (HT), a diverse field with multiple approaches in oncology, is poised to revolutionize cancer treatment. The potential to leverage the therapeutic benefits of heat offers inspiration for improved outcomes for cancer patients, whether as a standalone treatment or in combination with other therapies. The historical origin of hyperthermia, a field traced back to ancient Greek medicine when energy delivery to the body was limited, is a testament to its evolution. The discovery of electromagnetic energy transfers a couple of hundred years ago changed hyperthermia applications. This led to the development of numerous electromagnetic energy transports, including electric and magnetic fields, electromagnetic radiation: direct current, alternating current, radiofrequency (RF), microwave, and visible light. Non-electromagnetic applications such as focused ultrasound and hyperthermic perfusions were also developed. Diverse concepts supported these developments, all with the same goal: to selectively and homogeneously heat up the tumor at higher temperatures than the homeostatic control.

All electromagnetic interactions deliver energy to the biomaterials. The energy could be realized by heat and, consequently, temperature and electron excitation, making chemical reactions. These effects are naturally together because the bioelectromagnetic interactions partly modify compounds' chemical bonds and structure with electromagnetic forces. One part of the energy absorption heats the target, and the other makes the nonthermal interactions. The two energy components are used in two kinds of hyperthermic therapies in oncology. One direction was Arsene D'Arsonval (1851–1940), a French physician who successfully used nonthermally the electric and magnetic fields for oncotherapy [1]. The direct and alternating currents, mostly nonthermal, are applied with a combination of chemotherapy (electrochemotherapy) [2] in modern medicine. At the same time, the Danish physician Kristian Overgaard (1899–1976) focused on the thermal effect

alone [3]. The temperature approach was more straightforward and accessible to control and understand, and so became the mainstream of hyperthermia applications as a return to the ancient wishes with modern technologies. Due to the lack of a complete understanding of bioelectromagnetic processes, controversies were developing around it. Some measurements are misinterpreted, most claims have no data or only some subjective reports, and some results are shown only in individual cases. The wide variety of individual case reports makes it impossible to statistically evaluate those effects, which depends on the treated individuals. However, the debate about the divisive electromagnetic therapies is not useless. It provokes research and points out the physiological importance of electric currents, deriving many modern principles, including the control of the cellular effects [4] and modern neuroscience [5]. Even a general hypothesis of “biologically closed electric circuits” (BCEC) was formed based on the existence of intrinsic electric currents in the body, [6] [7], which idea is used in the treatment of malignant diseases [8] [9]. The pathological disorders [10] and wounds induce currents [11]. The spontaneous biological charge transfers caused by the tissue–repair process, [12] [13], and can be used as a noninvasive indicator of wound healing [14]. The biological effects of low-level, non-stationary magnetic fields have been observed [15] and adopted [16]. Among these effects, we may find some resonance character [17], demonstrating much experimental evidence from the field of ionic cyclotron resonance [18]. Several theoretical explanations of the ion cyclotron resonance have been developed [19]–[24]. The resonance effects are dominantly nonthermal and focus on the molecular excitations and structural changes, inducing desired signal pathways.

Bioelectromagnetic treatments are detached from hyperthermia, while the thermal and nonthermal components are applied in different shares in cancer distortion technical variants. For example, radiotherapy (RT) primarily uses nonthermal molecular breaking in DNA stands, and the thermal effects are negligible. On the contrary, hyperthermia is the opposite; it focuses on thermal energy, when the temperature is the single parameter that measures the dose, and the nonthermal energy is neglected in conventional evaluation. Such an approach to hyperthermia does not differentiate how which technique produces the heat. The exact temperature production is regarded as an identical treatment. However, it becomes increasingly evident that heat production is deeply connected to nonthermal bioelectromagnetic processes. Considering only the heat energy, the thermal homeostatic regulation, the stress reactions, the immunogenic aspects, enzymatic regulations, and many other molecular effects became one-sided, having only thermal dependence. On the other hand, the thermal calories are not equal to the same number of calories from food, inducing nonthermal chemical effects. Life is more chemical-based than thermal, so nonthermal chemistry cannot be neglected when evaluating bioelectromagnetic effects.

Energy absorption produces thermal and nonthermal processes. Thermal effects primarily involve heating tumors to temperatures typically between 39°C to 45°C, which may cause direct cytotoxicity, like protein denaturation. Still, it provides a general condition for optimization of the other nonthermal processes. The growing temperature increases the indispensable enzymatic reactions, but the enzymes are blocked over a thermal limit. Also, immune reactions have such thermal limitations. The nonthermal effects can significantly influence the overall efficacy and outcomes of hyperthermia treatments in oncology. The ionizing radiation like radiotherapy (RT) and the chemical interactions of drugs like chemotherapy (ChT), together with the many electromagnetic molecular effects and the immunogenic processes are nonthermal but use thermal conditions for higher efficacy (Figure 1). According to the Arrhenius law, thermal conditions increase sensitivity to radiation, and raising the reaction rate enhances chemotherapy efficacy.

The thermal background provides optimal efficacy for the nonthermal effects of ionizing radiation (radiotherapy), chemical reactions (chemotherapy), immune processes (immunotherapy), and electrohyperthermia, which uses the electric field to modify the molecular reactions.

2. DIFFERENCES BETWEEN THE HYPERTHERMIA TECHNIQUES

Different hyperthermia techniques may vary in their ability to induce these nonthermal biological effects effectively. Technical differences alter the synergy between thermal and nonthermal energy components.

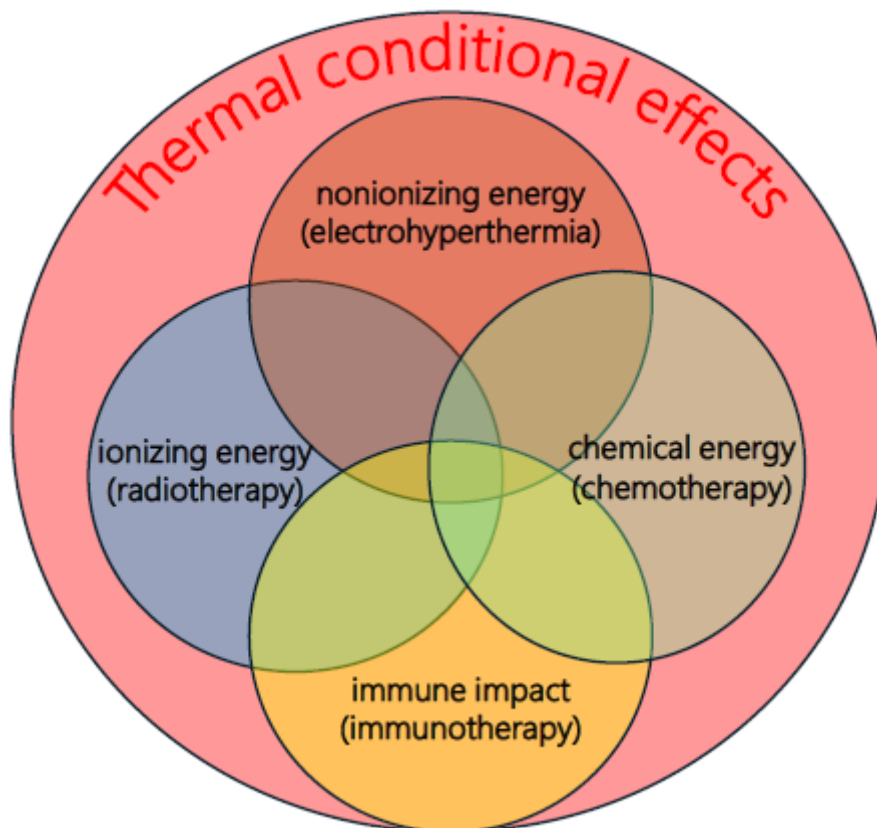


Figure 1. The thermal background (elevated temperature) provides the appropriate optimal conditions for using various nonthermal absorbed energies, including the electric field impact, ionizing and nonionizing processes, and the chemical changes by drugs.

The thermal and nonthermal processes are synergistically active in all applications, but the ratio of the two components may differ. The complex combination of the thermal and nonthermal effects can alter the physiological and biochemical properties (like blood flow, lymph transport, enzymatic processes, etc.). HT increases the microcirculation [25]–[34], and enhances the efficacy of the chemotherapies. The enhanced blood flow can improve the delivery of oxygen and ChT drugs to the tumor site [35]–[37]. Further support is that the hot drug is more reactive [38], providing excellent possibility for synergy, which is even more effective when considering accelerated drug metabolism and gained pharmacokinetic parameters. Furthermore, hyperthermia may activate the G0 phase of cell division, making these states reachable to reaction impacts. These advantages

can improve the efficacy of concurrent treatments and reduce the chances of treatment resistance.

Various electromagnetic and technical parameters produce hyperthermia effects, sometimes combined with mechanical actions like ultrasound. The technical variations depend on the various chosen designs (Figure 2). The technical parameters use the wide variability of the electromagnetic processes including the frequency, energy delivery, acting on selected volumes, and selective molecular focusing ability, the fiend could be formed in invasive form, and depends on the energy source, the energy coupling to the target and at the end the combined (complementary) applications with other treatments concomitantly or concomitantly next to each other.

Techniques differ in how the physiological changes can be manipulated effectively and how to improve treatment outcomes. The methods differ in activating the blood vessel permeability in tumors, which can enhance the delivery of chemotherapy drugs or oxygen to the tumor site. The nonthermal effects of various hyperthermia techniques involve a complex interplay of molecular excitations and biological responses triggered by cellular stress responses to elevated temperatures. These responses encompass a range of molecular pathways and signaling mechanisms that influence cell survival. The hyperthermia technique also varies by modulation of the immune response, potentially enhancing immune recognition and attacking cancer cells, increasing the therapeutic outcomes in oncology.

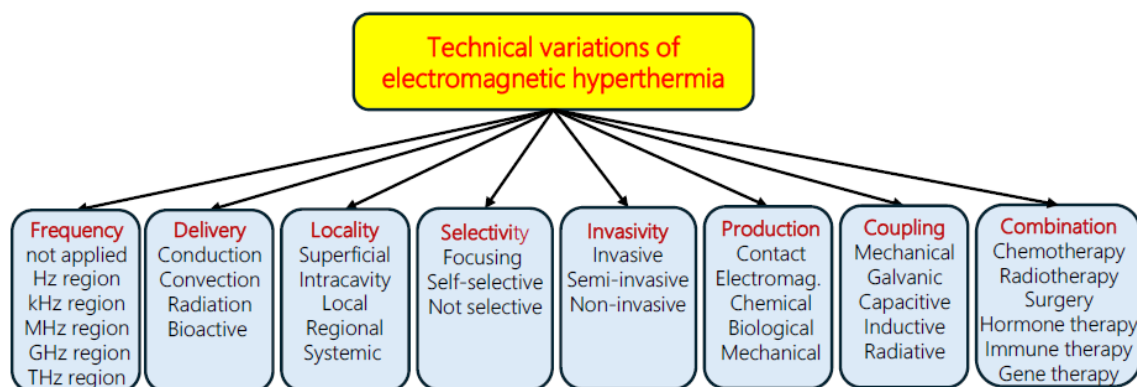


Figure 2. The possible technical parameters of electrohyperthermia methods. All shown categories have numerous subcategories and could be grouped in various technical solutions. The combination of the parameters allows hundreds of technical solutions.

The technical solution may vary the efficacy [39], modify the reaction rates measured by the Arrhenius plot [40], and develop different amounts of HSPs [41], different cell destruction [42] [43], different immune activity [44] [45], modifying the preclinical [46] and clinical results [47]. Various techniques differ in enhancing the synergistic effects of other cancer treatments, such as RT or ChT, by optimizing nonthermal mechanisms that may improve patient outcomes. The consequence of the variations appears in the essential differences between the characterization of the methods in Figure 3.

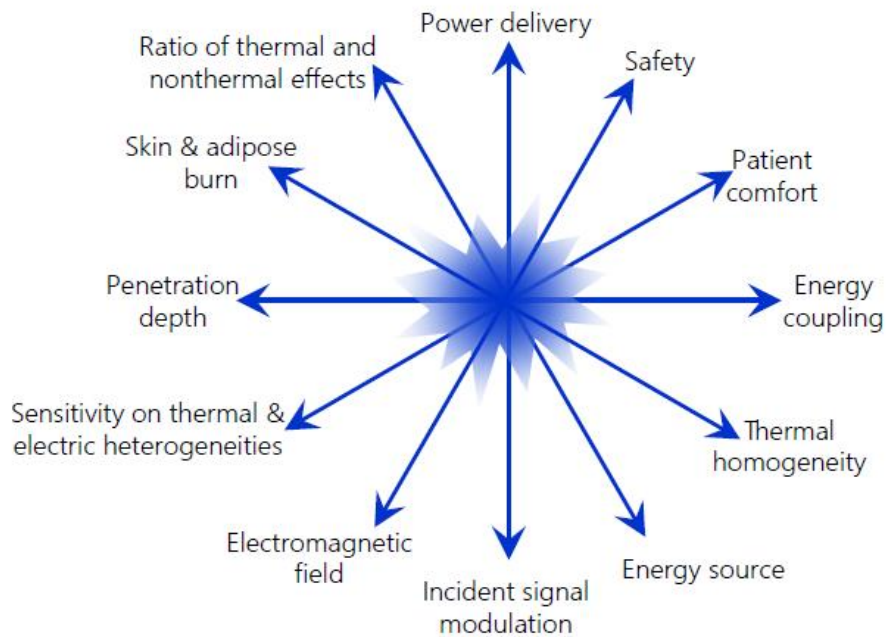


Figure 3. The primary technical and operational parameters of electromagnetic hyperthermia methods.

Understanding how thermal and nonthermal effects interact allows us to optimize basic treatment parameters such as temperature, duration of treatment, and integration with other therapies. Technical optimization maximizes therapeutic efficacy while minimizing adverse effects on healthy tissues. The optimum of the multivariant process is a complicated task, which is the primary intention for all hyperthermic designs. The optimum is a Nash equilibrium [48]. In this state, no further change of the technical parameters produces higher efficacy, and the contradictory interactions compensate each other in a balance. The equilibrium could be lost by changes in the conditions (different individuals, tumor stage and change, increasing temperature, etc.), which needs to repeat the optimizing process.

The differences in results between hyperthermia techniques can arise due to several other factors:

- 1) Different techniques may heat tumors to the same average temperature, but the distribution of heat within the tumor and penetration depth can vary. Some methods may be better at uniformly heating the tumor throughout its volume, a factor that can significantly influence treatment outcomes.
- 2) Variations in how well different techniques achieve and maintain the target temperature can affect treatment efficacy and safety.
- 3) The way heat is transferred to tissues can vary between techniques. Factors such as tissue perfusion (blood flow) and the thermal properties of tissues can impact how effectively the desired temperature is reached and maintained within the tumor.
- 4) Hyperthermia is often used in combination with other cancer treatments. The synergy between hyperthermia and these treatments may differ depending on the specific technique, influencing overall treatment outcomes.

- 5) Variability in patient anatomy and tumor characteristics can also interact differently with various hyperthermia techniques, affecting individual treatment responses.

The pivotal difference between the applied techniques is the ratio of thermal and nonthermal effects. The default effect is thermal. The nonthermal impact is a complementary additive to the thermal one. The nonthermal effects are induced nonionizing in the tumor microenvironment (TME) by the electric field. The complementary synergy of these effects works like the synergic addition of nonthermal ionizing radiation (radiotherapy) to the thermal impact alone, which is a well-proven combination [49]–[51]. The thermal effect and chemotherapy combination are similar to thermal and nonthermal amalgamation. The ChT focuses on chemical reactions, which are nonthermal, but the thermal conditions may accelerate the reaction rate and the transport of the chemical drugs [52]–[54].

The nonthermal effects are a larger category than hyperthermia. Ionizing radiation, nonionizing radiation, electromagnetic fields, and drug-induced chemo changes are all nonthermal (Figure 4). The thermal effects promote nonthermal processes, which could change the molecular bonds and make signal excitations by nonionizing energy as HT does. It does not have enough energy to ionize atoms or break chemical bonds. When the energy is as high as in the ionizing range, like in radiotherapy (RT), it breaks the molecular bonds. The synergy of these nonthermal effects works in thermo–radio–chemotherapy complementary applications. Thermally-induced nonthermal effects can stimulate anti-tumor immune responses by promoting antigen presentation, activating dendritic cells, and enhancing T-cell function. Changes in the TME, including increased infiltration of immune cells and modulation of immune checkpoints, can contribute to improved anti-tumor immunity.

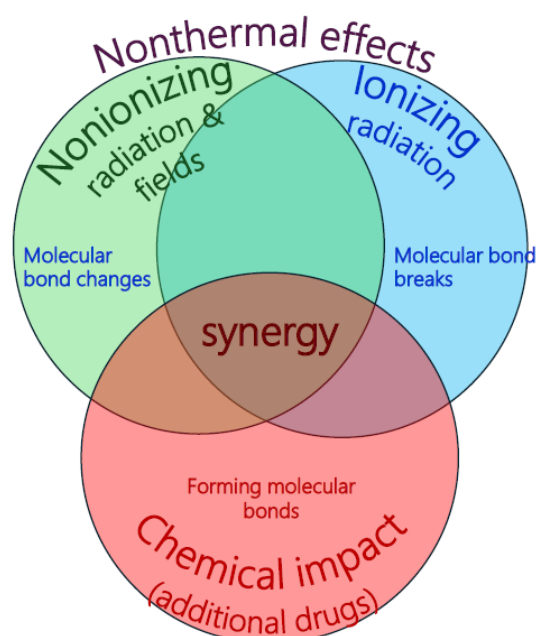


Figure 4. The ionizing and nonionizing processes have immense nonthermal effects on modifying molecular structures. Additional drugs make chemical impacts and form new molecules. Their synergy provides the basis for combining nonthermal HT effects with RT and ChT.

The essential biological differences between the electromagnetic hyperthermia techniques include numerous molecular and physiological changes (Figure 5). The consequence of the technical differences is the massive variation of the effects achieved with the applied method.

2.1. Thermal-Nonthermal Balance

Hyperthermia can induce various biological responses within cells and tissues beyond heating them. The optimal parameters are technique-dependent and determined by the interaction of thermal and nonthermal processes. They may enhance the overall effectiveness of HT as a treatment modality in oncology. The thermal effects induce a counter-reaction by thermal homeostasis, which tries to restore the equilibrium thermal conditions. The nonthermal effects modify the molecular bonds and may also have counter-reactions by natural compensations, like chaperone proteins. HT's thermal and nonthermal effects interact through various biological mechanisms, often synergizing to enhance treatment outcomes and interact complexly. The potential of hyperthermia to enhance treatment outcomes is significant, offering hope for improved patient outcomes. The thermal conditions provide the optimal situation for the nonthermal contribution to vasodilation and vasoconstriction, altering tumor blood flow. While heat directly damages cancer cells (thermal effect), it also releases HSPs into the tumor microenvironment (nonthermal effect) that can further sensitize cells to radiation or chemotherapy. Combining thermal and nonthermal effects can lead to complex biological responses within tumors. These responses may involve a balance between cytotoxicity (thermal) and immune activation (nonthermal), ultimately influencing tumor regression and clinical outcomes.

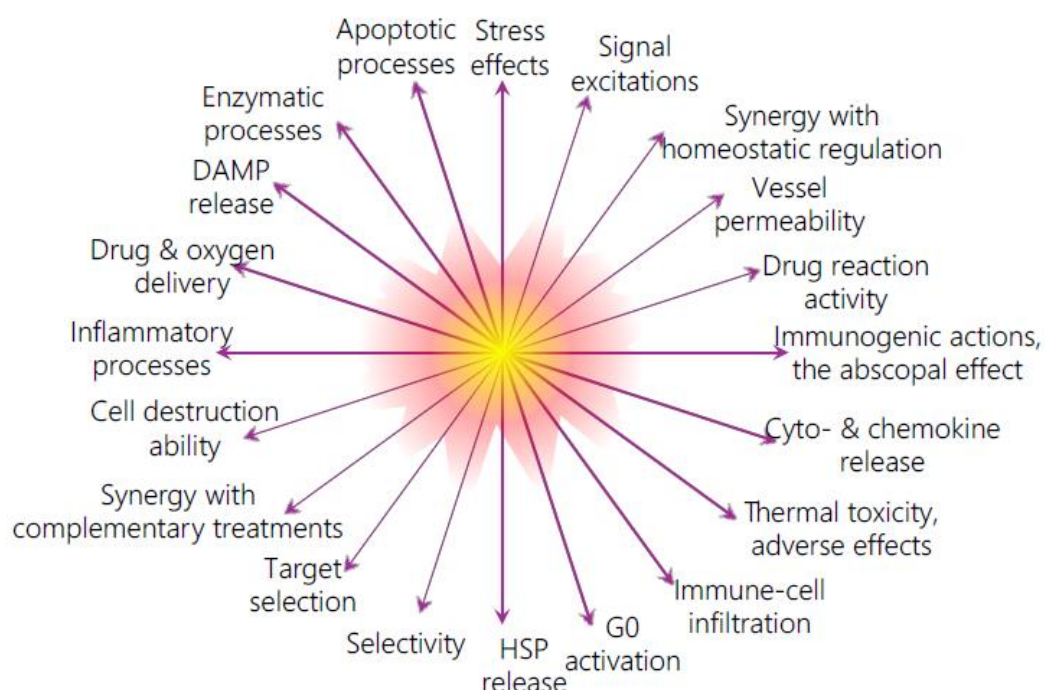


Figure 5. The important processes of mEHT.

The thermo-chemotherapy results in a better therapeutic effect, increasing the target specificity and reducing the systemic side effects [55] [56]. In some cases, low-dose chemotherapy could be

used [57] [58] with hyperthermia promotion; it is also applied in low-dose metronomic chemo-regulation [59]. Some key molecular excitations and processes involved in thermal and nonthermal effects are shown in Figure 6.

2.2. The Modulated Electrohyperthermia

The modulated electrohyperthermia (mEHT) tries to solve the optimal equilibrium of the thermal and nonthermal effects of the absorbed energy. There are numerous optimized parameters chosen from the electromagnetic possibilities (Figure 7).

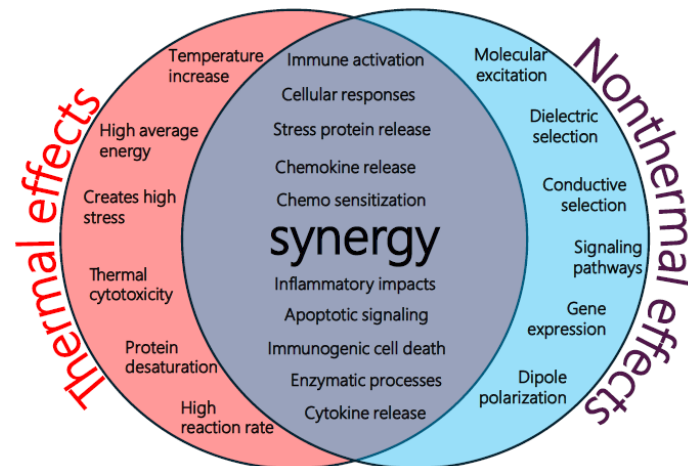


Figure 6. Differences and similarities interact in a synergy of energy absorption's thermal and nonthermal components.

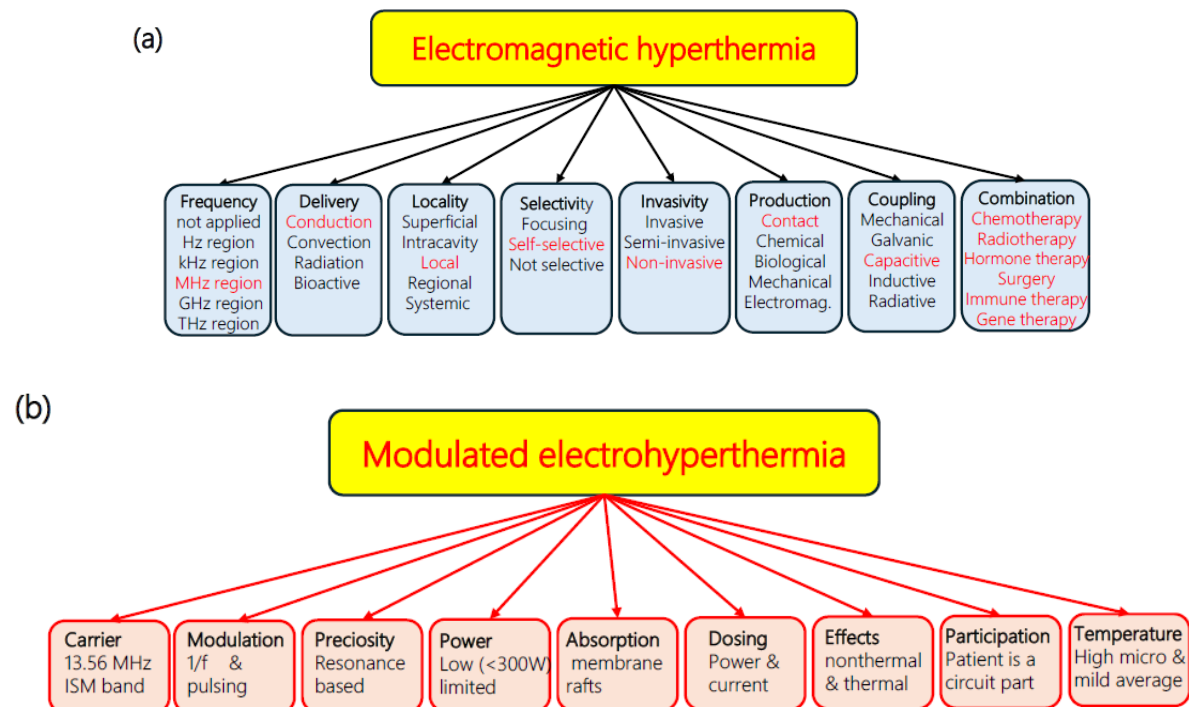


Figure 7. The selected parameters for mEHT. (a) Selection from the possibilities of electromagnetic hyperthermia (emphases with red letters); (b) The most important parameters of mEHT.

The chosen parameters of mEHT contribute to the synergy of thermal and nonthermal effects and harmony with natural homeostatic control. In details:

- 1) The carrier frequency is 13.56 MHz, which is in the β/δ frequency dispersion range. The β -dispersion is interfacial polarization [60], targeting the cancer region [61]. This frequency is optimal for selecting malignant tumors based on their extra electric conductivity [62], driving the radiofrequency (RF) current to this region. This behavior can also be used for diagnostics [63]. The cancer cells in the tumor behave autonomically and break the healthy network connections with other cells, which increases their dielectric constant (permittivity) [64]–[66], which allows their recognition [67] [68]. The 13.56 MHz is an ISM standard for hospitals, so it does not require extra shielding for operation. The high conductivity and dielectric behavior concentrate the energy absorption on the cell membrane [69]. The tumor microenvironment (TME) differs from the general extracellular matrix (ECM) [70], which helps the selection process.
- 2) The applied amplitude modulation is chosen from the low-frequency $\alpha\alpha$ -dispersion range, providing the appropriate extrinsic signaling for apoptosis and immunogenic cell death, forcing healthy homeostatic control [71].
- 3) The complete selection is resonance-based [72], accurately ensuring the optimum of the processes. Resonance is stochastic and fits the impedance matching and also the modulation [73].
- 4) Due to the molecular selection, the energy is not used to heat the entire tumor mass, which allows much lower incident power than in conventional hyperthermia applications [74]. This makes the method safer and causes fewer adverse effects, avoiding thermal toxicity (burns).
- 5) The overlapping $\delta\delta$ -dispersion [75] helps to select the water-bonded lipidprotein complexes in the glycoprotein lipid microdomains (rafts) [76]. The membrane rafts are enveloped in a nonconductive lipid membrane environment, so their relatively high conduction achieves their precise heating by increasing the mEHT treatment's heating preciosity [77]. Raft excitation is highly likely in these conditions and may trigger intracellular signals [78].
- 6) Due to the high preciosity and proper selection, the dosing does not need temperature control or enough energy (power, RF current) control in the treatment.
- 7) The thermal and nonthermal effects are synergistically optimized [74], giving the suitable condition for the signal transfers to develop a molecular pattern in an appropriate space-time form, releasing damage-associated molecular pattern (DAMP) in an immunogenic cell death (ICD) process [79].
- 8) The unique real-time tuning, which matches the patient's impedance to the treatment, increases the treatment accuracy [80]. The patient is an organic part of the RF resonant conditions, acting like one of the discrete elements of the electric circuit [81].
- 9) The mEHT is a kind of hyperthermia but a heterogeneous heating solution [82]. The selectively heated microdomains work like nanoparticle heating. The domains absorb high energy, having higher temperatures than their environment. They start to heat by conducting their vicinity, which produces a mild temperature level on average in the tumor.

The mEHT method has numerous publications discussing the above phenomena. The most comprehensive material could be found in two books, and some chapters of others dealing with this method [83]–[88].

3. PERSPECTIVES OF MODULATED ELECTROHYPERTHERMIA (MEHT)

The mEHT fits the latest trends in oncotherapy development [89] [90]. It is selective, safe and immunogenic.

3.1. Selective Application Using the Cancer's Electric and Thermal Differences (Biophysical Characters)

The main selectivity characters of mEHT [91]:

- Choses the tumor by high conductivity;
- Choses the tumor cells in the tumor mass by their autonomy;
- Choses the transmembrane proteins by their easy excitation;
- Absorbs the energy by homeostatic harmony.

The mEHT selectively attacks the malignant cells and mostly naturally kills them. It is essential because it allows for more effective and targeted treatment strategies, which can lead to better outcomes for cancer patients. When mEHT selectively targets the cancer cells, it can minimize damage to surrounding healthy tissues and organs, reducing the severity of side effects experienced by the patient. Selective targeting allows mEHT to focus more effectively on eliminating cancerous cells. This can enhance the overall efficacy of treatment by increasing the likelihood of killing cancer cells while sparing healthy ones. Cancer cells can develop resistance to treatments over time, making them harder to eliminate. The mEHT targets specific vulnerabilities of malignant cells and may reduce the likelihood of resistance developing, potentially improving long-term treatment outcomes. The selectivity of mEHT towards specific types of cancer cells can lead to more personalized treatment approaches. This could involve tailoring therapies based on the patient's cancer cells' genetic profile or other characteristics, potentially optimizing treatment effectiveness. The selectivity of mEHT can contribute to better patient outcomes by maximizing the therapeutic effect on cancer cells while minimizing harm to healthy tissues. This balance is crucial for improving cancer patients' survival rates and quality of life.

3.2. The Nonionizing Nonthermal Electric Field Application

The RF application of mEHT is nonionizing. It represents an innovative approach that offers significant advantages in terms of efficacy, safety, and patient-centered care compared to traditional ionizing radiation or chemotherapy. Continued research and technological advancements in this field hold promise for further improving outcomes and expanding treatment options for cancer patients. The mEHT offers several advantages that make it valuable in clinical practice. The main nonionizing nonthermal processes used by mEHT:

- Changes chemical processes without extra toxicity by using only natural processes;

- Guides the processes in harmony with homeostasis (note that it is applicable not only for oncology);
- Helps to reduce the side effects of conventional therapies by targeting the tumor;
- Improves the efficacy of chemo reactions and radio effects (note that it is applicable not only for oncology).

Nonionizing mEHT is delivered strictly localized. This localization helps minimize systemic toxicity and adverse effects commonly associated with systemic treatments like chemotherapy. The locally enhancing reaction rate of the chemo drug increases its efficacy, and the higher blood perfusion also increases its concentration. The locally higher concentration leaves less concentration for systemic adverse effects. It can be repeated multiple times if necessary, without cumulative toxicity. This flexibility can be beneficial for managing recurrent tumors or addressing residual cancer cells after initial treatment. The nanoscopic raft heating of mEHT offers precise control over the treatment area and depth, allowing clinicians to target tumors accurately. This precision helps to concentrate the therapeutic effect on the cancerous tissue while preserving nearby critical structures. The mEHT can often be combined with other treatment modalities such as surgery, radiotherapy, chemotherapy, or immunotherapy. This multimodal approach can synergistically enhance treatment outcomes by targeting different aspects of cancer biology or overcoming treatment resistance. The mEHT has demonstrated effectiveness across a range of cancer types and stages. They can be tailored to specific tumor characteristics, making them versatile in clinical oncology practice. It typically results in less discomfort, shorter recovery times, and improved overall quality of life for cancer patients.

3.3. Immunogenic Effects, Tumor Vaccination

The immunogenic effects of mEHT therapy refer to the treatments' ability to stimulate the body's immune system to recognize and attack cancer cells. This approach offers several distinct advantages in the treatment of cancer. The immunogenic effects in cancer therapy represent a promising avenue for improving treatment outcomes, expanding treatment options, and potentially achieving long-term remission or even cures for certain types of cancer. The main immunogenic effects of mEHT [92]:

- Activates the innate and adaptive immune system by recognizing the cancer cells;
- Produces tumor antigen presentation for killer T-cells;
- Attacks the cancer cells all over the body in micro and macro metastases (abscopaleffect);
- Works like tumor vaccination (patented).

The immunogenic effect of mEHT can specifically target cancer cells based on their unique molecular markers or antigens, potentially sparing healthy tissues from damage. The induced immunogenic cell death releases unharmed molecules with information about the cancer cells, which was hidden for immune surveillance until this. This targeted approach also enhances the specificity of treatment. The mEHT immunogenic cell death (ICD) can induce a durable antitumor response. Once activated, the immune system can continue recognizing and eliminating cancer cells, providing long-term benefits. ICDs can activate immune cells throughout the body, enabling them to seek out and destroy cancer cells wherever they may be present, including micro and macro metastatic sites (abscopal effect) [93] [94]. This systemic effect enhances the treatment's ability to address widespread disease. The likelihood of developing resistance to mEHT therapy decreases over time is minimal. The mEHT can overcome these resistance mechanisms by

targeting different pathways or enhancing immune surveillance. The mEHT can be combined with other treatments that can synergistically improve treatment outcomes by simultaneously targeting multiple aspects of cancer biology. The mEHT immunogenicity is automatically tailored to individual patients based on their immune profile and the specific characteristics of their cancer. This personalized approach also may improve treatment efficacy and reduce unnecessary side effects. The mEHT-induced ICD may successfully treat cancers that were previously difficult to treat with conventional therapies. The mEHT develops a tumor-specific vaccination, which can induce immunological memory. This means that the immune system retains a memory of the cancer cells, protecting against recurrence or future metastases.

4. CONCLUSIONS

The perspectives of hyperthermia, especially the modulated electrohyperthermia, have multiple possible ways for future realization.

- 1) Hyperthermic therapies must increase their focus on the target, avoid unwanted hot spots in healthy tissues, and limit heat spreading by natural heat conditions. The mEHT, with its molecular selectivity on the membrane rafts of malignant cells, optimizes the selectivity of energy absorption.
- 2) Hyperthermia must harmonize thermal and nonthermal processes to choose the best signal pathways to destroy the tumor. The mEHT optimizes the molecular actions and signals excitations with a synergy of the thermal and nonthermal processes.
- 3) The rapid development of immuno-oncology affects all modern oncology treatments. Hyperthermia must fit this new trend. The mEHT, which induces immunogenic cell death, induces immunogenic effects and could be well harmonized with other immunotherapies.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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COMPARISONS OF COMPUTER SIMULATIONS AND EXPERIMENTAL DATA FOR CAPACITIVE HYPERTHERMIA USING DIFFERENT SPLIT-PHANTOMS

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ABSTRACT

Introduction: Several positive clinical trials have demonstrated that capacitive hyperthermia (CHT) improves the effectiveness of radiation therapy for the treatment of various cancer entities. However, the ability of CHT to induce significant heating throughout the body is under debate. Objectives: To perform a pilot study involving comparisons of computer simulations and experimental data using different split-phantoms to validate hyperthermia treatment modeling for pre-planning for a clinical CHT system and to investigate the feasibility of split-phantom measurements in capacitive hyperthermia.

Materials and methods: The CHT system EHY-2030 (Oncotherm, Budapest, Hungary) was used. The system provides two electrode sizes, but only the smaller electrode, indicated as D200 electrode, was investigated in this pilot study. Horizontally and vertically splittable, different multi-slice phantoms with dielectric material properties simulating muscle and electrically low conductive fat were produced and heated. During the heating procedure, temperature-time curves were measured, and thermal images were captured. Specific absorption rate values were derived from the temperature rise (TR) values. Concomitantly, computer field simulations utilizing a detailed CAD-based model of the CHT system were performed using the simulation platform Sim4Life and compared with measurements.

Results: For the investigated electrode D200 the system power of 75W was applied, which is half of the maximum power of 150W and lies in the range of usual values for this electrode applied in patient treatments in our clinic. For 75W, a heating of 3.6°C in 6min in a depth of 1cm in an agar-based, muscle tissue-equivalent phantom was achieved. The addition of a 1cm thick, synthetic, low dielectric fat layer reduced the TR up until a depth of 8.5cm by on average around 38% (from 8.5cm onwards the absolute local TR is similar, deviations are $\leq 0.1^\circ\text{C}$). In terms of point-to-point absolute SAR comparison (without any normalization), up to a depth of 11cm in the phantoms central vertical plot, the simulation differs from the measured TR points by on average 25% (ranging from 7% to 36%) for the homogeneous phantom and by on average 43% (ranging from 26% to 60%) for the inhomogeneous phantom.

Conclusion: Computer simulations and experimental data were compared for the CHT system EHY-2030 using the D200 electrode, applying a thermal imaging technique for different vertically splittable phantoms. This pilot study data can be used as a guidance regarding the expected heating for this commonly used electrode size but also to further elucidate the significance of non-thermal anticancer effects. Further studies are needed for different sizes and geometries of electrodes and phantoms.

1. INTRODUCTION

Hyperthermia based on radiofrequency (RF) electromagnetic fields (EMF) is widely used in cancer treatment. For instance, hyperthermia using radiative multiple antenna or waveguidebased devices was shown to increase the effects of chemotherapy and radiation therapy in randomized controlled trials [1–4]. The anticancer effects caused by the achieved temperature increase are pleiotropic including increased oxygenation and perfusion, inhibition of DNA repair and immune effects among others [5–7]. A different approach to couple EMF to the patient body is the use of

electrodes. When electrodes are used at relatively low frequencies, i.e., below ~30MHz, the EMF energy is mainly capacitively coupled to the tissue. This technique is, therefore, known as capacitive hyperthermia (CHT) and the electrodes together with the tissue between the electrodes are part of the electric circuit. An essential characteristic of capacitive coupling is that the direction of the E-field is predominantly perpendicular to the contact surface between the electrode and the skin, as well as the subcutaneous layers. This leads to a high specific absorption rate (SAR) inside and a high-power loss at these layers, as the EMF jumps there due to the interface conditions (continuity of the normal electric flux density). This, together with the limited steering abilities when compared to radiative devices, is the reason why, subcutaneous fat poses a hindrance for capacitive devices, when intending to treat deep seated tumors [5,8].

The adjustable variables for CHT are: the choice of the electrode's size, the positioning of the electrode (including the electrode's water bolus) with respect to the treatment region and the geometry of the patient's surface, the insertion of additional water bags between the water bolus and patient's skin for better electromagnetic coupling and additional surface cooling and a proper surface cooling (also preferably including a coolant circulation for the cooling of the electrode's metallic structures) to prevent too high temperatures in the water bolus and on the skin surface and thus to enable the application of a sufficiently high energy dose [5,8]. Lastly, a separate variable is the (optional) use of amplitude modulation, as further described later on.

Several positive, randomized controlled trials demonstrated that CHT improves the effectiveness of radiation therapy for the treatment of various cancer entities [9–17]. Still, there are also studies showing no benefit from the addition of hyperthermia to radiotherapy [18]. Notably, apart from Minnaar et al. most of those trials were performed in Asian countries, where the patients generally are of a slimmer habitus. It is therefore unclear whether similar results can be achieved in western countries, where the population shows a differing body composition, since fat layers are proven to limit deep heating [8,19–21]. After all a higher thermal dose of $CEM_{43T90} > 7 \text{ min}$ (CEM_{43T90} is CEM_{43} calculated for T_{90} , T_{90} being the temperature, which is exceeded in 90% of the tumor; CEM: cumulative equivalent minutes at 43°C) was already shown to be a significant predictor of biochemical disease-free survival [22].

While CHT is accepted as a state-of-the-art method for superficial hyperthermia [23], its acceptance regarding its excellence in inducing significant heating throughout the body toward deeper tumor localizations is still under debate. Recently, efforts were started to develop simulation programs for several CHT systems [24] but not including the system presented in this paper. In general, comprehensive phantom studies that include measurements are rather sparse for CHT. For instance, the present authors were involved in the extended phantom measurement study for the Celsius 42 system that was used by our Berlin hyperthermia group [18]. For the system presented in this work no such study has been performed so far. One of the CHT systems is the hyperthermia device EHY-2030 (Oncotherm, Budapest, Hungary), which is clinically used in our department. In this paper, in order to compare computer simulations and experimental data using this system, we present a pilot study involving a quantitative comparison of split-phantom measurements and computer simulations for a detailed CAD-based model of the device. Concerning the fact that both, simulations and measurements, were compared in such a detailed and quantitative way, this is our knowledge the first such a study worldwide for a clinical CHT system so far. This pilot study data can be used to validate hyperthermia treatment modeling for pre-planning for a clinical CHT system and to investigate the feasibility of split-phantom

measurements in capacitive hyperthermia. Furthermore, this study may be used as a guidance regarding the expected heating for this commonly used electrode size but also to further elucidate the significance of non-thermal anticancer effects. Another goal of our work was the implementation of a framework for future treatment planning for CHT. For this, further similar studies are planned for different sizes and geometries of electrodes and phantoms.

2. MATERIALS AND METHODS

2.1. Hyperthermia device

The aforementioned hyperthermia device EHY-2030 consists of the (electrically grounded) therapy bed, on which the patient lies and the upper “active” electrode, which is connected to the amplifier channel and can be placed onto the patient’s treatment region by moving the electrode’s rotatable arm. The operating frequency of the system is 13.56MHz.

The upper electrode can be detached from its arm and exchanged for a different-sized electrode. The commercial system provides two electrode sizes but only the smaller electrode, indicated as ‘D200 electrode’, was investigated in this pilot study. The D200 electrode has a diameter of around 20cm and the system allows to drive this electrode with a maximum forward power of 150W. The system may also be connected to a bigger electrode of around 34cm diameter (indicated as ‘D300 electrode’), that is driven by a maximum forward power of 250W. Further investigations involving the bigger electrode are planned in the next future, however this requires bigger phantoms, which were not available for this pilot study.

2.2. Phantom rationale and design

Different multi-slice phantoms were produced similar to those recommended by the European Society for Hyperthermic Oncology (ESHO) guidelines for superficial hyperthermia [23]. At this point, it is worth noting that the main aim of this study was not to judge the thermal effectivity of this system via strictly fulfilling the procedures and temperature limits defined by the ESHO guidelines. This study has to be understood as a pilot study to check the feasibility of the phantom production and use of the splitting technique, as well as to perform comparisons with numerical results (see Section 4). However, with respect to the geometry requirements, we designed our experiments based on the information provided by the ESHO guidelines. According to the guidelines, the phantom should be twice the size of the used applicator electrode in the lateral dimensions and the thickness of the phantom should be at least three times the expected penetration depth. With a cylindrical phantom of a diameter of 45cm and a thickness of 22cm, we fulfilled both phantom size requirements for the D200 electrode. The large D300 electrode would require larger phantoms in lateral directions and thus the construction of bigger casting molds, so we decided to focus on the D200 electrode in this pilot study.

The thickness of 22cm was made up of a 20cm thick agar cylinder in the center with two additional 1cm thick layers, which enclose the phantom on the top and at the bottom. For comparison, two cases for these 1cm thick layers were investigated: one with an in-house produced synthetic fat layer and another with a layer made up of agar gel. To the phantom with the synthetic fat layer, we here refer to as the ‘inhomogeneous’ phantom (details see Section 2.4) and the phantom with the

agar-based layer is in the following referred to as the 'homogeneous' phantom (details see Section 2.3). With the phantoms' increased total thickness and the additional 1cm thick layers, made up of a synthetic fat layer in the inhomogeneous phantom, not only at the top but also at the bottom of the phantoms, we also aimed to mimic a real patient's condition.

Furthermore, similar to the phantom construction recommended in the ESHO guidelines our multi-slice phantoms were splittable in their vertical plane and the horizontal layers could also be removed, thus allowing temperature measurements using a thermal camera through the central vertical and a horizontal plane between the 1cm thick layer and the rest of the phantom.

With good accuracy, the phantom's density was assumed to be $\rho = 1000\text{kg/m}^3$ [21,25]. It had a weight of around 35kg.

For the fabrication of our phantoms, using a 3D print technique, we constructed a special casting mold, in which the heated agar gel was filled and left to cool down (Figure 1). The casting mold was 3D-printed part by part using polycarbonate. The single parts were then screwed together with stainless steel screws. The casting mold was designed to produce one half of the cylindrical, muscle tissue-equivalent phantom allowing us to then put two halves together to receive a complete phantom and, after the heating experiment, to easily split it vertically in two halves for the thermal imaging. The phantoms were assembled without any additional, disturbing layer or material in between.

The additional 1cm thick layers were cast in two cooking pans (diameter of 45cm). This was done to parallelize the casting of the different phantom parts and was especially important for the synthetic fat layers, since the reached temperatures in their production would have been too high for the polycarbonate casting mold to withstand.



Figure 1. (a) View from the planar side onto the casting mold and (b) view from above onto the casting mold.

2.3. Multi-slice, homogenous phantom

For the creation of the 20cm thick muscle tissue-equivalent phantom, that forms the basis of both the homogeneous and inhomogeneous phantom, the recipe [25] recommended in the ESHO guidelines for superficial hyperthermia [23] was followed (Table 1).

First the sodium chloride was dissolved in the deionized (DI) water. The solution was heated until 50–60°C. Next, the agar was slowly added while stirring the mixture and heating it at 80–90°C for around 5min. After that, the mixture was slowly filled into the casting mold up to the 20cm thickness mark. It was then left to cool down, solidify and was ready to be used in approximately 24h. This procedure was repeated for the second half of the agar cylinder. For the homogeneous phantom a third repetition of the cooking process was done for the remaining two 1cm thick agar layers, where the heated agar gel was evenly filled into two cooking pans up to the 1cm mark. Finally, the two agar layers were positioned below and on top of the 20cm thick muscle tissue-equivalent phantom to form the complete multi-slice, homogeneous phantom with a thickness of 22cm.

2.4. Multi-slice, inhomogeneous phantom

For the multi-slice, inhomogeneous phantom, a recipe from a recent publication [26] was applied (Table 2) [26].

It must be emphasized that continuous stirring of the mixture was necessary during all the following steps. First, the oil and the glycerol were mixed at room temperature in a cooking pot placed on a powerful enough cooking plate. It was made sure that no air bubbles were present in the resulting gel. After around 10min, a uniform emulsion was obtained. As next, the emulsion's temperature was gradually increased to 130°C. Once 130°C was reached, the ethylcellulose was gradually added until it was dissolved. Next, the pot was partly covered to minimize the dispersion of vapors, and the temperature was increased to 170°C. Finally, the mixture was evenly poured into the two cooking pans up to the 1cm thickness mark and was left to cool down.

After cooling down, the two synthetic fat layers were ready to be used and placed on top and at the bottom of the 20cm thick muscle tissue-equivalent phantom to create a finished inhomogeneous phantom with a thickness of 22cm (Figure 2).

Table 1. Quantities used in the production of the muscle tissue-equivalent phantom following the publication from [25].

Substance	Quantity
Sodium chloride	3.3 g
DI water	Around 956 g
Agar powder	40 g

Table 2. Quantities used in the production of the synthetic fat layer following the publication from [26].

Substance	Quantity in wt%
Canola oil	36
Ethylcellulose	7
Glycerol	36

2.5. Heating procedure

For the temperature measurements the two halves of the muscle tissue-equivalent phantom were placed on the applicator table with the 1 cm thick additional layers, either the agar or the synthetic fat layer, at the bottom and at the top of the phantom (Figures 3 and 4). The upper electrode was then positioned centrally on the phantom. It was taken care that full contact was established between the device and the phantom. The electrodes coolant temperature was equal to the room temperature being 20 °C.

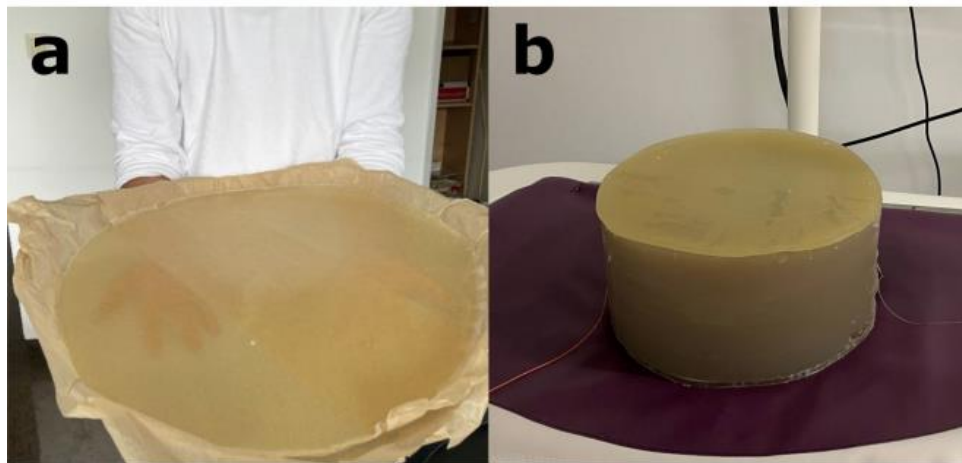
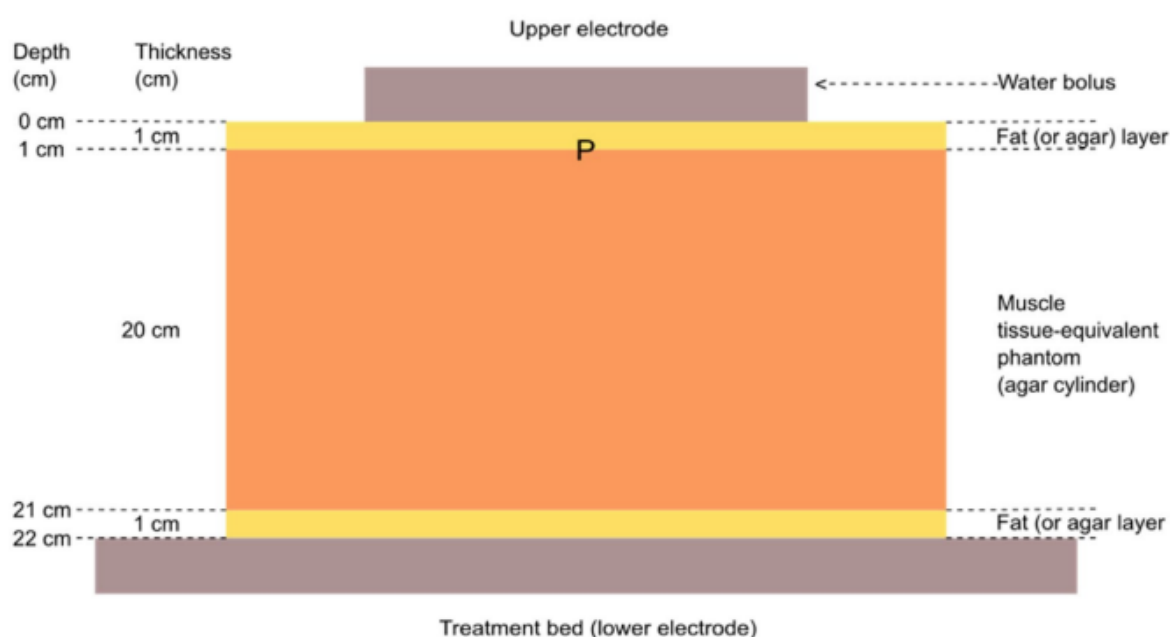


Figure 2. (a) In-house produced, synthetic fat layer with a thickness of 1cm and a diameter of 45cm and (b) assembled inhomogeneous phantom, consisting of the muscle tissue-equivalent phantom (diameter 45cm, height 20cm) in the center and two additional 1cm thick synthetic fat layers on the top and at the bottom.



Figure 3. Assembled inhomogeneous phantom during a heating procedure with the EHY-2030. The phantom has 4 parts. A circular agar cylinder (diameter 45 cm, height 20 cm) is built from

two vertically splittable, half-cylindrical parts (darker color). This cylinder is horizontally bounded by two 1 cm thick top and bottom layers of circular shape (diameter 45 cm, lighter color), consisting of either agar gel (homogeneous phantom) or synthetic fat (inhomogeneous phantom), like in this picture. The metallic structures of the upper electrode (D200) are located behind the cylindrical (white) plastic cover and are cooled by a system with a circulating coolant at a constant temperature of around 20 °C. Between the cooled metallic electrode parts and the phantom, there is a cylindrical bolus that contains DI water and is surrounded by a silicon cover (purple color) and a special conductive textile material. The water in this bolus is not part of the system's circulating coolant. For further detailed information, see, Figure 4.



For the heating procedure we chose 6min, which is also the heating time recommended by the ESHO guidelines (note again, that the main aim of this paper was not to judge on the thermal effectivity of this system via a strict fulfillment of the procedures and temperature limits defined by the ESHO guidelines, see the discussion section). We applied 75W, which is half the D200 electrode's maximum power output of 150W (75W lies in the range of usual average values applied in clinical treatment for this electrode in our clinic). Inside the phantom the TR is with a good accuracy proportional to the applied power, thus the results for maximum power output of 150W can be, if necessary, easily obtained by multiplying the TR result by a factor of 2. Detailed information about the validity of this 'power scaling' for measurements can be found in Section 2.8, below. Prior to each measurement the phantom, and all thermally relevant system parts like the electrode's water bolus, were left to cool down again to room temperature. For further details, see also the legends of Figures 3 and 4.

2.6. Temperature measurement procedure

The utilized thermal camera was the FLIR 8E-TX (Teledyne FLIR, Frankfurt am Main, Germany) with a heat sensitivity (NETD) of $<0.05^{\circ}\text{C}$ and a temperature accuracy of $\pm 2\%$. The used thermal probe is the fiber optic temperature sensor OTG-MPK5 (opSens-Solutions, Quebec, Canada) with a temperature accuracy of $\pm 0.3^{\circ}\text{C}$ and a reaction time of 225ms. The time between each registered measurement point of the thermal probe was set to the lowest possible interval of 2.8s. It was

aimed to position the probe in a depth of 1cm. This was done by pinching it in between the two halves of the muscle tissue-equivalent phantom (Figure 4) (the depth of 0cm is the top surface of the fat (or agar) layer, i.e., the contacting surface to the electrode's water bolus).

Before the heating procedure, temperature measurements were performed. Thermal images were taken of the horizontal plane in 1cm depth after removing the upper layer, and of the central vertical plane by splitting the phantom open in its two halves. The temperature probe measurements were continuously monitored 1min before the heating and during the heating.

After six minutes of heating, the upper electrode together with the upper 1cm thick layer was swiftly removed and again a thermal image of the horizontal plane was captured. Next, the phantom was quickly split open and a thermal image in the vertical plane through the phantom was captured. It was taken care and ensured that those measurements were completed within 15s.

After the phantom had fully cooled down, it was assembled again for the next heating procedure. For each of two phantoms, two experiments were performed, respectively.

The thermal images were assessed with FLIR Thermal Studio (Teledyne FLIR, Frankfurt am Main, Germany). In that software, the fit between the image and the overlaid thermal image was further adjusted and optimized.

After that the measurement points, for which the temperature was assessed in the software, were positioned on an axis through the captured horizontal or vertical plane. Finally, the TR was obtained by subtracting the given temperature in two corresponding measurement points in the thermal image before and after the treatment.

2.7. Determination of the TEFS and TEPD from the TR

The thermal effective field size (TEFS) is defined as the area within 50% of the maximum TR, observed in the horizontal plane at 1cm depth in the phantom [23].

The thermal effective penetration depth (TEPD) is defined as the depth, at which the TR drops to 50% of the maximum TR measured in 1cm depth [23].

2.8. Determination of the SAR from the TR (relationship between SAR and TR)

2.8.1. Conductive heat transfer equation

In the case of our measurements in non-perfused phantoms the relationship between TR and SAR is described with good accuracy by the conductive heat transfer equation, which can be derived from the bioheat transfer equation (BHTE), originally formulated by Pennes [27]. Thus, starting, for instance, from the BHTE notation used in Equation (1) in [28], we obtain the following conductive heat transfer equation, which with good accuracy describes temperature behavior in our phantoms during heating:

$$\rho \cdot c \cdot \frac{\partial T}{\partial t} - \nabla \cdot (\kappa \cdot \nabla T) \approx \rho \cdot \text{SAR}. \quad (1)$$

where t is the time in seconds, T is the temperature in kelvins with the known initial condition $T(t=0)$, ρ is the density in kilograms per cubic meters, c is the heat capacity in watts times seconds per kilograms per kelvins, κ is thermal conductivity in watts per meters per kelvins, and SAR is the specific absorption rate in watts per kilograms. In general, the SAR in Equation (1) can be time-dependent, but in our experiments, it is with good accuracy a constant value during heating.

2.8.2. Power scaling

The temperature distribution T in Equation (1) can be formulated as a sum of a 'basal' temperature T_0 (T before heating) and a temperature rise distribution TR . As shown in [28] in the Equations (5a) and (5b), when certain conditions are fulfilled, the relationship between TR and SAR is linear, i.e., if, for instance, twice the power is applied and consequently each SAR value is doubled in any point, as a result the TR value in these points is doubled, too. We refer to this linear relationship in this paper as 'power scaling'. We used the principle of power scaling for determining the TR values that would be obtained by utilizing the maximum power (150W) from our TR measurements done with half the system's maximum power (75W).

In the case of numerical calculations, the validity of this power scaling procedure is explained in [28] in Equations (5a) and (5b). In the case of measurements, the power scaling principle is valid for any power used, when, firstly, the material constants like the thermal conductivity and heat capacity do not change their values during heating, and secondly, the boundary conditions (BC), as well as the initial conditions are the same for any power applied (basal T_0 remains the same). As to the first necessary condition: as shown in [29], for the temperatures in the range of 20–30°C, covering the temperatures reached in our experiments, the material properties can be assumed as temperature-independent. As to the second condition: This condition is of course 'automatically' fulfilled because we change only the power applied to the system, but the initial conditions (certain phantom temperature distribution before heating) are by definition the same. Furthermore, the same BC for any applied power means, that in the case of a Dirichlet-type BC the same temperature at the phantom's surface is present (for instance a room temperature of constantly 20°C), and/or that in the case of the Cauchy-type heat transfer BC, the same exterior temperature (in our case 20°C) and the same (and temperature-independent) value of the outward heat transfer coefficient are given. Again, with good accuracy, it can be assumed that in the range between 20°C and 30°C the outward heat transfer coefficient remains constant. Moreover, the room temperature is constantly held at 20°C via the air conditioning in our clinic.

Another good illustration of the validity of power scaling is the general possibility of the use of the Green's function (GF) representation for Equation (1). The GF describes the temperature distribution caused by an instantaneous, local heat pulse [30]. Again, the power scaling is possible if the GF is dependent only on the geometry and material parameters, but not on the temperature, i.e., particular material properties like the heat capacitance and thermal conductivity must not be temperature-dependent in the thermal range of interest (20–30°C). In conclusion, power scaling can be used with good accuracy for our phantoms because of the linearity of the problem described in Equation (1) under the assumption of temperature-independent material properties.

2.8.3. Formulation without conduction term for the SAR determination from the TR ('temperature-rise method'). Restrictions for comparison between measurements and calculations

A very often applied way to create a simple and direct relationship between SAR and TR values that allows a direct determination of SAR from TR (and vice versa) is an assumption, that in some points of the investigated domain, the term $\nabla \cdot (\kappa \cdot \nabla T)$ in Equation (1) can be neglected [31]. Consequently, in these points the heat conduction Equation

(1) gets reduced to a simple formula: $c \cdot \frac{\partial T}{\partial t} \approx \text{SAR}$. When SAR

values do not change during heating, $\frac{\partial T}{\partial t}$ can be replaced by

$\text{TR}/\Delta t$, where Δt is heating time (in our case $\Delta t = 6 \text{ min}$). Subsequently, the following simple formula (often referred to as 'temperature rise method') can be obtained for the determination of the SAR from the TR:

$$\text{SAR} \approx c \frac{\text{TR}}{\Delta t} \quad (2)$$

Points, for which the approximation in Equation (2) is appropriate, may be used for determination of SAR values from measured TR values. In our case, these experimentally derived SAR values were compared to the calculated SAR values, as shown in Section 3.3. For points that are situated in our agar-based muscle tissue-equivalent phantom material, for which we applied Equation (2), we inserted the heat capacitance $c = 4184 \text{ J/kg} \times \text{C}$ [25]. The procedure described in Equation (2) is true with good accuracy for points that are not situated near electromagnetic and/or thermal boundaries, like the phantom's surface or interfaces between different media like between the agar gel and the synthetic fat. At such interfaces, firstly, electromagnetic properties may abruptly change and thus the SAR may 'jump' or exhibit sharp 'hot spots' that may lead to steep temperature gradients and hence may generate a heat conduction. Secondly, thermal material properties may also abruptly change and therefore a measured TR value in a point near an interface may be related not only to one single medium, but to two media, situated on both sides of the interface, and this relationship may be very complex or even unclear. Consequently, we applied the formula in Equation (2) only to those points that are located in a distance of at least 1cm from thermal and/or electromagnetic boundaries/interfaces.

2.9. Simulation procedure

For the phantom measurements, corresponding simulations for the calculation of RF-EMF were performed using the simulation platform Sim4Life (ZurichMedTech, Zurich, Switzerland). Due to a relatively low operating frequency of 13.56MHz and as a result a very large wavelength in comparison to the geometry size, the influence of the magnetic field can be neglected and therefore a fast Sim4Life solver, using a quasi-static electromagnetic approximation of the Maxwell's equations, was applied.

Accurate computer-aided design (CAD) models of the applicator device and its electrode were provided by the manufacturer (Oncotherm, Budapest, Hungary) (Figure 5). Moreover, computed

tomography (CT) scans of the phantoms were acquired, from which accurate 3D models were derived. This was done by exporting the CT scans as polygons and converting them into 3D voxel models with an equal resolution to the CT data, using the software Plastimatch (The General Hospital Corporation, Massachusetts, USA) and an in-house written code in Matlab (The MathWorks, Inc., Massachusetts, USA).

Finally, the simulation results were exported and the SAR for the different depths were read out in a .xlsx file. After this, the percentual difference between the measured and simulated SAR at the different depths was determined.

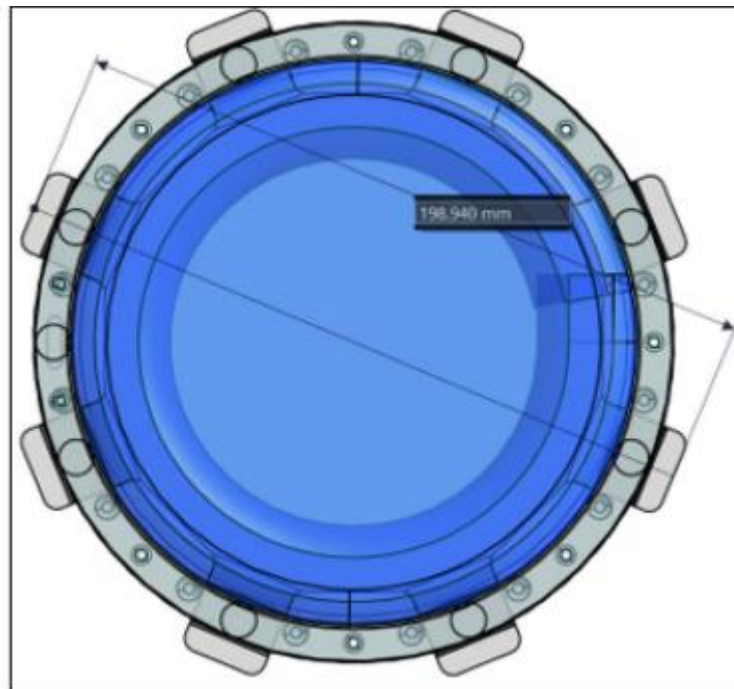


Figure 5. Computer-aided design (CAD) model of the electrode D200 of the hyperthermia system EHY-2030, containing complex conductive textile and bolus parts attached to a metal ring structure.

3. RESULTS

3.1. Dielectric property assessment

Prior to the measurements, we needed to assess the accurate dielectric properties of the used materials in our measurements, to insert them into our simulation settings (Table 3).

For this, the DAKS-12 (SPEAG, Zurich, Switzerland) was acquired and implemented into our experimental workflow. For Table 3, there is worth noting, that with an electrical conductivity of 0.54S/m and a relative permittivity of 78.52 we were well in the range of the reported values in the literature [23, 25].

Concerning our low dielectric synthetic fat layer, the original publication introducing our followed recipe already reported lower dielectric values than the ones of real fat for our investigated

frequency range, with the electrical conductivity being 36 times lower and the relative permittivity being 1.5 times lower than the literature values for real fat tissue [26]. The measurements of our own fat layers showed even lower dielectric properties, with the electrical conductivity being smaller by a magnitude of two powers of ten and the relative permittivity being 31% lower than those reported in [26].

3.2. Temperature measurements

With an applied power of 75W for 6min, the measurements show a TR of 3.6°C in 1cm depth in the central plot of the homogeneous phantom (Figure 6). The TR for the inhomogeneous phantom at that depth in the central plot is 2.3°C. As aforementioned, however, 75W is only half of the maximum power available for this electrode. Thus, to make a statement for the device's maximum capabilities, those results must be scaled to the device's maximum power output of 150W, resulting in a TR of 7.2°C for the homogeneous and 4.6°C for the inhomogeneous phantom (for power scaling, see Section 2.8.2). For the homogeneous phantom at 75W a TR of 1.8°C was observed up to a depth of between 2.3 and 2.9cm and for 150W up to between 4.1cm to 4.8cm.

For the inhomogeneous phantom, this is the case up to a depth of between 1.6 and 2.3cm for an applied power of 75W and up to a depth of between 2.9 and 3.5cm for an applied power of 150W.

A TEPD of 2.3 to 2.9cm was measured for the heating procedure with the homogeneous and 2.9 to 3.5cm for the inhomogeneous phantom. The averaged TEFS are 178cm² for the homogeneous phantom and 254cm² for the inhomogeneous phantom (Figure 8a and b and Supplementary Figure 1).

Table 3. Measured dielectric properties with the DAKS-12. SD is the standard deviation.

Material	Electrical conductivity (S/m)	Relative permittivity
Muscle tissue-equivalent phantom (agar gel) (16 averaged measurements)	0.54 (SD 0.015)	78.52 (SD 3.246)
Synthetic fat layer (6 averaged measurements)	0.514×10^{-3} (SD 1.158×10^{-5})	11.34 (SD 0.224)
DI water	0.1758×10^{-3}	76.74
Silicone	0.3275×10^{-4}	3.41
Leatherette (on the applicator table)	0.12×10^{-3}	4.13

Obviously, the synthetic fat layer causes that some parts of the energy are directed in the horizontal direction instead of penetrating toward the perpendicular direction, which can be observed by comparing the TEFS for both phantoms.

Note that for the TEFS determination of the homogeneous phantom the TR values in the 1cm deep horizontal plane were used (Supplementary Figure 1). In the case of the inhomogeneous phantom a depth of 1cm would be at the interface between the upper 1cm thick fat layer and the muscle tissue-equivalent phantom. Since the TEFS and TEPD determination has to be done in reference to the max TR 1cm deep inside the muscle tissue-equivalent phantom, the measured TR values in the vertical plane at 2.3cm depth and thus at 1.3cm depth inside the muscle tissue-equivalent phantom were utilized in the case of the inhomogeneous phantom (Figure 8b).

On one hand the TR measurement recorded with our thermal probe for the inhomogeneous phantom, being 2.4°C (SD 0.43), corresponds well to the one measured with the thermal camera. The thermal probe measurement for the homogeneous phantom on the other hand, being 2.08°C (SD 0.23), corresponds well to the measured one with the thermal camera in a depth somewhat deeper than 1cm. This was due to the fact that for the placement of the thermal probe, no fixated catheter inside the phantom was utilized. This was done to prevent disruptive effects on the EMF by additional material boundaries. Instead, the thermal probe was pinched in between the two muscle tissue-equivalent phantom halves. This made an accurate positioning in 1cm depth difficult and led to the thermal probe's position being somewhat deeper than 1cm.

3.3. Comparison to the simulations

Figure 7 shows the comparison between the measurements and the simulations in terms of absolute SAR (as pointed out in Section 2.8.3, for measured data, the SAR is derived via the TR by Equation (2) using the thermal capacity of agar gel of $c=4184\text{J/kg}\times\text{C}$). The simulations refer to the absorbed power of 75W in the total calculation domain, which was the power used for measurements as shown in Figure 6 in terms of the TR. Note that for the inhomogeneous phantom measurements, only from a depth of 2.3cm onwards up until 18.5cm the TR derived SAR values can be used for a robust comparison. As explained at the end of Section 2.8.3, we apply the formula in Equation (2) for this comparison only to those points that are located in a distance of at least 1cm from thermal and/or electromagnetic boundaries/interfaces, with the interface between fat and agar being situated at 1cm and 21cm depth. The results are presented in terms of absolute SAR (i.e., not nominalized to any maximum value like the SAR at depth 1cm, etc.). At the depths of 1cm, 1.6 and 2.3cm the simulated SAR is higher than that derived from measured TR values via Equation (2) by 7% (3W/kg), 18% (6.8W/kg) and 24% (7.4W/kg) for the homogeneous phantom. For the inhomogeneous phantom at the depth of 2.3 the simulated SAR is 26% (6W/kg) higher. The absolute SAR difference on average is only 3.7W/kg (ranging from 0.6 to 8W/kg) for the homogeneous phantom across all measurement points and 4.3W/kg (ranging from 0.6 to 8.7W/kg) for the inhomogeneous phantom from 2.3cm on up until 18.5cm depth. Up to a depth of 11cm the simulation differs from the measured TR points by on average 25% (ranging from 7% to 36%) for the homogeneous phantom and by on average 43% (ranging from 26% to 60%) for the inhomogeneous phantom. From a depth of 13.5cm on, the simulations start to differ from the measurements by more than 60%. Since the SAR in the depth is very small, even very little differences of around 1.5W/kg lead to a high relative difference. Thus, with increasing depth inside the phantom, the absolute SAR difference becomes more meaningful.

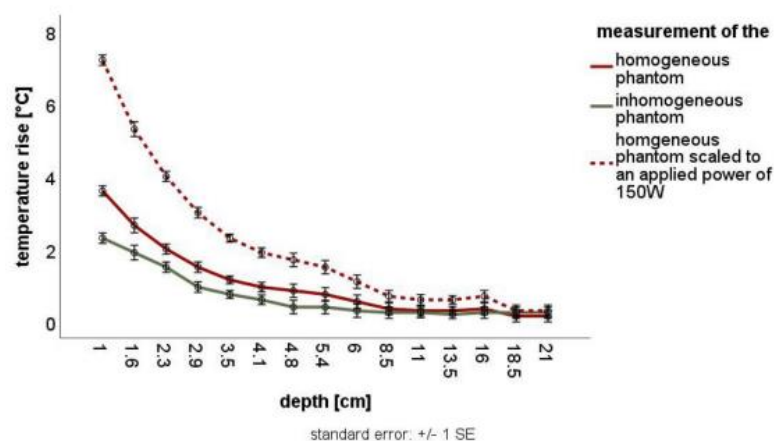


Figure 6. Measured TR for the D200 electrode driven using 75W (half of the maximum possible power output of 150W for this electrode) in the central vertical plane (along the rotational phantom's symmetry axis perpendicular to the electrode) through the phantoms in dependence of the depth from the top to the bottom of the phantoms. Two experiments were done for each phantom. (the depth of 0 cm is the top surface of the fat (or agar) layer of 1 cm thickness, i.e., the contacting surface to the electrode's water bolus. Thus, the depth of 1 cm is the interface between the top fat (or agar) layer of 1 cm thickness and the vertically splittable circular agar cylinder (diameter 45 cm, height 20 cm). The depth of 21 cm is the interface between the circular agar cylinder and the bottom fat (or agar) layer of 1 cm thickness.).

The simulation of the homogeneous phantom shows a TEPD of around 3.3cm and the simulation of the inhomogeneous phantom a TEPD of around 4.1cm, which is for every phantom type around 0.5cm higher than the TEPD determined from measurements. For the depth of 0 and 0.5cm (these points are not shown in Figure 7) and an applied power of 75W, the simulation shows a SAR of 59 and 54W/kg for the homogeneous phantom. For the inhomogeneous phantom, around 131W/kg is simulated at both, 0 and 0.5cm depth. Note, furthermore, in Figure 7, that, for the homogeneous and inhomogeneous phantom, the initial SAR decay (in the depth between 1 and 2.3cm) is steeper for the measured data than for the calculations.

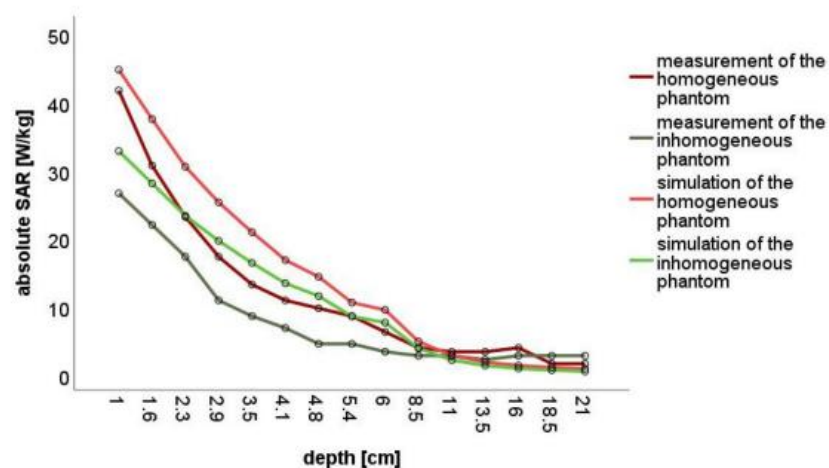


Figure 7. Absolute SAR through the central vertical plane (along the rotational phantom's symmetry axis perpendicular to the electrode) of the phantoms derived from measurements via Equation (2) and the corresponding simulations in dependence of the depth for a total power of 75W. The standard error for the measured values is given in Figure 6. For the depth of 0 and 0.5 cm (these points are not shown in Figure 7) and an applied power of 75W, the simulation shows a SAR of 59 and 54W/kg for the homogeneous phantom. For the inhomogeneous phantom, around 131W/kg is simulated at both, 0 and 0.5 cm depth. Note that for the inhomogeneous phantom measurement only the depicted SAR values from a depth of 2.3 cm onwards up until 18.5 cm are reliable as a distance of at least 1 cm to the fat/agar interface is required for Equation (2) to be applicable. (the depth of 0 cm is the top surface of fat (or agar) layer of 1 cm thickness, i.e., the contacting surface to the electrode's water bolus. Thus, the depth of 1 cm is the interface between the top fat (or agar) layer of 1 cm thickness and the vertically splittable circular agar

cylinder (diameter 45 cm, height 20 cm). The depth of 21 cm is the interface between the circular agar cylinder and the bottom fat (or agar) layer of 1 cm thickness.).

Figure 8a) TR homogeneous phantom														
depth	horizontal position [cm]													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
1	0.15	0.35	0.65	1	2.9	3.85	3.75	2.95	3.2	2.6	0.65	0.45	0.3	0.2
2.3	0.05	0.1	0.25	0.65	1.25	1.95	2.05	1.5	1.25	0.9	0.35	0.1	0.05	0.05
3.5	0	0.05	0.15	0.35	0.6	0.95	1.1	1	0.75	0.45	0.1	0.1	0	0
6	0.05	0.05	0.15	0.25	0.4	0.55	0.55	0.6	0.5	0.25	0	0	0.05	0
8.5	0	0.05	0.1	0.3	0.3	0.4	0.45	0.4	0.35	0.1	0	0	0	0.2
11	0	0.05	0.1	0.25	0.2	0.35	0.3	0.35	0.25	0.15	0	0.05	0	0
13.5	0	0.05	0.2	0.3	0.2	0.25	0.35	0.3	0.2	0.2	0.05	0	0.05	0
16	0.1	0.15	0.25	0.3	0.2	0.3	0.3	0.25	0.2	0.2	0.05	0	0	0
18.5	0.2	0.25	0.15	0.2	0.15	0.15	0.15	0.2	0.15	0.15	0.1	0.15	0.1	0.05
21	0.2	0.2	0.15	0.25	0.15	0.15	0.1	0.15	0.15	0	0.05	0.1	0.05	0.3

Figure 8b) TR inhomogeneous phantom														
depth	horizontal position [cm]													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
1	0.5	0.55	0.6	0.95	1.8	2.95	2.5	2.55	2.6	2	1	0.65	0.4	0.2
2.3	0.3	0.35	0.4	0.6	1.3	1.75	1.45	1.6	1.45	0.9	0.55	0.4	0.35	0.3
3.5	0.25	0.3	0.3	0.4	0.7	0.85	0.7	0.85	0.7	0.4	0.3	0.3	0.3	0.2
6	0.25	0.25	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.35	0.3	0.2
8.5	0.25	0.2	0.3	0.3	0.25	0.3	0.3	0.2	0.3	0.25	0.3	0.3	0.3	0.2
11	0.15	0.25	0.3	0.25	0.25	0.3	0.25	0.3	0.35	0.3	0.35	0.3	0.3	0.2
13.5	0.2	0.2	0.3	0.25	0.25	0.25	0.25	0.25	0.3	0.3	0.35	0.3	0.3	0.2
16	0.25	0.25	0.3	0.25	0.25	0.25	0.25	0.25	0.3	0.2	0.3	0.3	0.3	0.2
18.5	0.25	0.25	0.3	0.25	0.25	0.25	0.2	0.2	0.25	0.25	0.3	0.2	0.3	0.25
21	0.25	0.25	0.3	0.25	0.3	0.25	0.2	0.3	0.25	0.2	0.3	0.25	0.25	0.2

Figure 8. (a and b) Matrices of the measured TR for the D200 electrode driven using 75W (half of the maximum possible power output of 150W for this electrode) in the central vertical plane (along the rotational phantom's symmetry axis perpendicular to the electrode) through the homogeneous (a) and inhomogeneous (b) phantom in dependence on the depth from the top to the bottom of the phantoms. Two experiments were done for each phantom. (the depth of 0 cm is the top surface of the fat (or agar) layer of 1 cm thickness, i.e., the contacting surface to the electrode's water bolus. Thus, the depth of 1 cm is the interface between the top fat (or agar) layer of 1 cm thickness and the vertically splittable circular agar cylinder (diameter 45 cm, height 20 cm). The depth of 21 cm is the interface between the circular agar cylinder and the bottom fat (or agar) layer of 1 cm thickness. The average standard error for the two measurements done is 0.115 for the homogeneous phantom (a) and 0.27 for the inhomogeneous phantom (b).

3.4. Comparisons on central vertical planes

In Figures 8a and b and 9a–d comparisons are shown in the form of 'matrices' for the measurements and simulations for both phantoms in their central vertical planes, depicting the TR and the SAR, as further insightful information can be drawn from them.

For both, the simulations and the measurement of the two phantoms, a decrease in TR and SAR can be observed from the center of the upper electrode onwards to the bottom and to the sides of the phantom. In both the y- and the x-axis the maximum TR and SAR is achieved in a 4.5cm radius around the center of the upper electrode.

Referring to the presented matrices, similar to the results from 3.3, a better agreement between measurements and simulations is given up to a depth of 11cm.

For an evaluated radius of 7.5cm the average relative difference between simulated and measured SAR [derived from the measured TR via Equation (2)] is 25% for the homogeneous and 42% for the inhomogeneous phantom. The absolute difference is on average 3.61W/kg for the homogeneous and 3.12W/kg for the inhomogeneous phantom.

When increasing the evaluated radius to 10.5cm, the relative difference is 35% for the homogeneous and 58% for the inhomogeneous phantom. The absolute difference then is 3.18W/kg for the homogeneous and 2.79W/kg for the inhomogeneous phantom.

From a depth of 13.5cm on and with a larger evaluated radius reaching the average relative difference even exceeds 100%. Since the SAR in the depth and toward the edges of the phantom is small, this still translates to rather small absolute differences (Figure 9).

4. DISCUSSION

The comparisons between numerical and experimental data using split-phantoms were performed for the EHY-2030 system utilizing the D200 electrode at 75W (which is half of the maximum power of 150W for this electrode and lies in the range of usual values for this electrode applied in patient treatments in our clinic) results in a TR of 3.6°C in 6min in 1cm depth in the muscle tissue-equivalent (homogeneous) phantom in its central plot in the central vertical plane. For the maximum power of 150W, when applying the power scaling procedure explained in Section 2.8.2, this is with good accuracy equivalent to a heating of 7.2°C in 6min in a depth of 1cm. The results for the homogeneous phantom in its central plot in the central vertical plane show that at the maximum power output of 150W a TR of at least 1.8° in 6min (corresponding to a SAR of around 20W/kg, which is a value comparable to moderate values used in deep hyperthermia applications, see, e.g., Table 1 in [31]) can be obtained up to a depth of around 5cm. For the inhomogeneous phantom, this is the case up to a depth of 3.5cm.

To achieve a TR of 6°C, in the central plot in the central vertical plane, a depth of 1, 2 and 3cm a power output of around 125, 194 and 319W would be needed for the D200 electrode in the case of the homogenous phantom. For the inhomogeneous phantom the necessary power outputs would be around 196, 265 and 500W, respectively. The maximum electrode power of 150W is about 5 times smaller than the typically used power range for multi-antenna systems. Consequently, using a similar electrode with a higher maximum power, the SAR level would rise appropriately. Note that more contemporary, commercially available electrodes can apply a power of >200W.

In deeper regions, we measured a TR and SAR below the aforementioned values, but it does not necessarily prove that a therapeutic effect can be excluded in such deep-seated regions. It has been described preclinically [32] and it has been confirmed by our group in cell experiments that non-thermal anticancer effects of RF-EMF based CHT exist, especially when amplitude modulation is applied [33–35]. These non-thermal anticancer effects may, in addition to the described depth-dependent TR due to CHT, explain the positive outcome in several randomized controlled trials [9–16]. Further research and clinical trials are required to study the anticancer effects of CHT for instance in body regions, where a TR is hard to be realized or even currently unfeasible treatment sited such as the liver and the brain. We have launched pilot studies to investigate this issue, which will also involve field simulations of patient models (Clinicaltrials.gov identifiers NCT06140875 and NCT05991102).

The synthetic fat layer impairs the heating in the tissue underneath, up until a depth of 8.5cm inside the phantom in its central plot in the central vertical plane (from 8.5cm onwards the absolute local TR is similar, deviations are $\leq 0.1^{\circ}\text{C}$). At the measurement point in 1cm depth inside both phantoms in their respective central plot in the central vertical plane., the TR is 36% lower for the inhomogeneous phantom when compared to the homogeneous phantom. At depths of 1.6, 2.3, 2.9, 3.5 and 4.1cm the TR is 28%, 25%, 37%, 35% and 37% lower, respectively, for the inhomogeneous phantom.

From the presented matrices (Figure 8a, b and 9a–d) it can be concluded, that in difference to Beck et al. [21], no hotspots are observed at the edge of the electrode or more precisely near the edge of electrode's water bolus that was contacting the phantom's top surface. Note, however, that a different capacitive system with a different electrode geometry was used in that study.

In addition, we were able to establish field simulations, and our SAR simulations show encouraging results, at least up to a depth of 11cm, with a difference of on average 25% for the homogeneous and 43% for the inhomogeneous phantom in their central vertical plots. This may enable our routine toward a simulation environment in Sim4Life feasible for further in silico studies with patient models.

Considering the whole central vertical plane up to a depth of 11cm and an evaluated radius of 10.5cm there is a difference of on average 35% for the homogeneous and 58% for the inhomogeneous phantom.

Figure 9a) calculated SAR homogeneous phantom														
depth	horizontal position (cm)													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
1	1.74	4.07	7.55	11.62	33.69	44.72	43.56	34.27	37.17	30.20	7.55	5.23	3.48	2.32
2.3	0.58	1.16	2.90	7.55	14.52	22.65	23.81	17.43	14.52	10.46	4.07	1.16	0.58	0.58
3.5	0.00	0.58	1.74	4.07	6.97	11.04	12.78	11.62	8.71	5.23	1.16	1.16	0.00	0.00
6	0.58	0.58	1.74	2.90	4.65	6.39	6.39	6.97	5.81	2.90	0.00	0.00	0.58	0.00
8.5	0.00	0.58	1.16	3.49	3.49	4.65	5.23	4.65	4.07	1.16	0.00	0.00	0.00	2.32
11	0.00	0.58	1.16	2.90	2.32	4.07	3.49	4.07	2.90	1.74	0.00	0.58	0.00	0.00
13.5	0.00	0.58	2.32	3.49	2.32	2.90	4.07	3.48	2.32	2.32	0.58	0.00	0.58	0.00
16	1.16	1.74	2.90	3.49	2.32	3.49	3.49	2.90	2.32	2.32	0.58	0.00	0.00	0.00
18.5	2.32	2.90	1.74	2.32	1.74	1.74	1.74	2.32	1.74	1.74	1.16	1.74	1.16	0.58
21	2.32	2.32	1.74	2.90	1.74	1.74	1.16	1.74	1.74	0.00	0.58	1.16	0.58	3.49

Figure 9b) calculated SAR inhomogeneous phantom														
depth	horizontal position (cm)													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
1														
2.25	3.49	4.07	4.65	6.97	15.10	20.33	16.84	18.59	16.84	10.46	6.39	4.65	4.07	3.49
3.5	2.90	3.48	3.48	4.65	8.13	9.87	8.13	9.87	8.13	4.65	3.48	3.48	3.48	2.32
6	2.90	2.90	3.48	3.48	3.48	3.48	3.48	3.49	3.49	3.49	3.49	4.07	3.49	2.32
8.5	2.90	2.32	3.48	3.48	2.90	3.48	3.48	2.32	3.49	2.90	3.49	3.49	3.49	2.32
11	1.74	2.90	3.48	2.90	2.90	3.48	2.90	3.49	4.07	3.49	4.07	3.49	3.49	2.32
13.5	2.32	2.32	3.48	2.90	2.90	2.90	2.90	2.90	3.48	3.48	4.07	3.49	3.49	2.32
16	2.90	2.90	3.49	2.90	2.90	2.90	2.90	2.90	3.48	2.32	3.49	3.49	3.49	2.32
18.5	2.90	2.90	3.48	2.90	2.90	2.90	2.32	2.32	2.90	2.90	3.48	2.32	3.49	2.90
21														

Figure 9c) simulated SAR homogeneous phantom														
depth	horizontal position [cm]													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
1	0.04	0.38	1.44	6.18	34.98	45.34	44.89	45.04	45.72	30.56	5.54	1.40	0.38	0.08
2.3	0.08	0.45	1.38	5.02	17.69	27.96	30.40	30.38	27.60	16.16	4.61	1.33	0.40	0.11
3.5	0.14	0.44	1.29	3.98	10.97	17.95	20.73	20.65	17.55	10.24	3.74	1.25	0.50	0.15
6	0.24	0.48	1.10	2.49	5.06	7.84	9.43	9.38	7.66	4.87	2.43	1.09	0.49	0.26
8.5	0.34	0.54	0.96	1.74	2.92	4.14	4.89	4.87	4.08	2.87	1.73	0.98	0.56	0.38
11	0.45	0.59	0.88	1.34	1.96	2.55	2.91	2.90	2.53	1.95	1.36	0.91	0.62	0.48
13.5	0.49	0.62	0.83	1.12	1.46	1.77	1.94	1.94	1.76	1.47	1.15	0.88	0.68	0.54
16	0.45	0.64	0.81	1.01	1.21	1.37	1.46	1.46	1.36	1.21	1.04	0.89	0.76	0.57
18.5	0.74	0.69	0.83	0.95	1.06	1.14	1.18	1.17	1.12	1.05	0.96	0.91	0.94	0.78
21	1.48	0.76	0.90	0.94	0.97	0.99	1.00	0.99	0.97	0.94	0.90	0.88	1.08	0.00

Figure 9d) simulated SAR inhomogeneous phantom														
depth	horizontal position [cm]													
[cm]	-19.5	-16.5	-13.5	-10.5	-7.5	-4.5	-1.5	1.5	4.5	7.5	10.5	13.5	16.5	19.5
0.5	0.00	0.00	0.02	0.27	82.79	130.97	130.72	130.76	131.01	48.35	0.18	0.01	0.00	0.00
1														
2.3	0.06	0.32	1.16	4.61	16.90	22.96	23.39	23.41	22.85	15.38	4.08	1.08	0.30	0.07
3.5	0.09	0.33	1.07	3.56	14.99	14.99	16.42	16.37	14.68	9.21	3.23	1.00	0.31	0.09
6	0.16	0.36	0.89	2.14	4.42	6.67	7.82	7.76	6.47	4.16	2.00	0.83	0.34	0.15
8.5	0.25	0.41	0.76	1.42	2.39	3.36	3.93	3.89	3.27	2.28	1.34	0.71	0.38	0.23
11	0.35	0.46	0.69	1.05	1.52	1.97	2.23	2.21	1.92	1.46	0.99	0.63	0.41	0.30
13.5	0.40	0.49	0.65	0.86	1.10	1.32	1.45	1.44	1.30	1.06	0.81	0.59	0.43	0.33
16	0.40	0.53	0.62	0.74	0.87	0.98	1.03	1.03	0.96	0.85	0.71	0.58	0.46	0.34
18.5	0.79	0.59	0.61	0.67	0.72	0.76	0.78	0.78	0.75	0.71	0.65	0.58	0.52	0.40
21														

Figure 9. (a–d) Comparison in vertical planes between absolute SAR derived via Equation (2) from Figure 8a and b (a and b, respectively) and the corresponding simulated absolute SAR, for a total power of 75W (c and d, for the homogeneous, and the inhomogeneous phantoms, respectively). Note that for the inhomogeneous phantom (b and d) the depth values of 1 cm and 21 cm exactly indicate the positions of the interfaces between agar and fat. Therefore, no SAR comparison is performed for these depth values (for details of validity of Equation (2), see Section 2.8.3). For simulated SAR, additionally, the SAR directly inside the fat layer (depth 0.5 cm) is reported (d).

The heating abilities of the EHY-2030 system favorably compare with those of another CHT system previously investigated by our group, Celsius42 [21]. In fact, for the homogenous phantom, when considering the used power, electrode size and distance between the electrodes, in a normalization to the equal power density approach, the EHY-2030 system shows almost a 2.7-fold higher SAR in a depth of 1cm [21]. A difference in favor of the EHY-2030 can also be observed, when comparing the SAR in the inhomogeneous phantoms in 1cm depth scaled to an equal surface power density. Here, the SAR for the EHY-2030 is almost 60% higher than for the Celsius42.

Those points can be contextualized with the earlier mentioned clinical trial utilizing the EHY-2030. There, the authors report that a temperature of 42.5°C was set as the target temperature during the treatments, and the energy output from the first electrode was compared to the energy received from the 2nd electrode, and, using algorithms, the device calculated the intratumoral temperature.

Contrary, in an older study performed on a smaller amount of 11 patients with deep-seated pelvic tumors with a different CHT device operating at 13.56MHz, the temperature was measured with thermal probes inserted into the tumor and normal tissue [8]. Their subcutaneous fat layers were 1.5cm (anteriorly) and 2.1cm (posteriorly) thick on average. There, for 60% of the patients, the intratumoral temperature was lower than 40°C and thus lower than what would be considered as ‘classic hyperthermia’. Fitting well to our simulations for the inhomogeneous phantom, the authors

of this study report that in 53% of the treatments the maximum temperature was measured in the subcutaneous fat. This further highlights the crucial role of surface cooling, which was also shown to reduce the temperature of the subcutaneous fat tissue [8].

Moreover, it has to be noted that the BMI was no predictor of LDC in that South African clinical trial. This also adds to the line of thought that sole heating of the tumor might not be the only physical parameter responsible for the positive results [12].

When considering a physical variable to measure the aforementioned effects on a cellular level, the current density may be a suitable parameter. The current density is a vector parameter, whereas the SAR is a scalar one. However, as the current density is proportional to the E-field rather than to the square E-field, its relative behavior can be approximately calculated based on the square root of the SAR values of our measurements. We propose further investigation, for instance to study a potential correlation between current density and cancer outcome.

Finally, it is worth noting that, even if we designed our pilot study using similar geometry and heating time to those proposed by the ESHO guidelines, the main aim of this study was not to judge on the thermal effectivity of this system via a strict fulfillment of the procedures and temperature limits defined by the ESHO guidelines. This study has to be understood as a pilot study to check the feasibility of the phantom production and use of the splitting technique, as well as to perform comparisons with numerical results. Furthermore, as only one electrode was used in this study, the final characterization of the system cannot be claimed. Moreover, we do not claim to decide if the system is 'thermally efficient' or not. Note that the system's driving power limitation with respect to both electrodes is rather conservative and, as it was already stated above, there are, in principle, no technical limitations for using higher powers than 150W for the D200 electrode, if necessary (note that the exchangeable bigger D300 electrode of the system is capable of using 250W). In general, hyperthermia is a multifaceted treatment so full clinical usefulness of a complex medical device is not only dependent on a single number like 'ability of heating of 6 degrees Celsius in 6min'. Moreover, measurements were taken in phantoms instead of patients in which, for instance, blood perfusion adds to the cooling and cooling inhomogeneity. On the other side, a synthetic 'fat' layer was used for the inhomogeneous phantom in our paper that is not equal to the real fat tissue, and thus higher SAR values may be expected in real fat. Again, an open question is if these higher SAR values would 'automatically' lead to higher temperature, when blood perfusion is present. Further studies should be performed to fully characterize the heating abilities of CHT devices, using both phantom and real patient set-ups.

5. CONCLUSION

Computer simulations and experimental data were compared for the CHT system EHY-2030 using the D200 electrode, applying a thermal imaging technique for different vertically splittable phantoms. In this pilot study the system power of 75W was chosen, which is half of the maximum power of 150W and lies in the range of usual values for this electrode applied in patient treatments in our clinic. For 75W, a heating of 3.6°C in 6min in a depth of 1cm in the central plot in the central vertical plane in an agar-based, muscle tissueequivalent phantom was achieved. The addition of a 1cm thick, synthetic, low dielectric fat layer reduced the TR in the central plot in the central vertical plane up until a depth of 8.5cm by on average around 38% (from 8.5cm onwards the absolute local

TR is similar, deviations are $\leq 0.1^{\circ}\text{C}$). Measured results have been compared with simulations. For a full characterization of this system, further studies involving the large electrode D300 and larger phantoms suited for this electrode are needed. Moreover, further studies should be performed to fully characterize the heating abilities of CHT devices, using both phantom and real patient set-ups.

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Disclosure statement

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Data availability statement

The data that support the findings of this study are available from the corresponding author, [PG], upon reasonable request.

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PULSED MODULATION IN ELECTRO-HYPERTHERMIA

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ABSTRACT

Modulated electro-hyperthermia (mEHT) is one of the novel oncological treatments with many preclinical and clinical results showing its advantages. The basis of the method is the synergy of thermal and nonthermal effects, similar to the thermal action of conventional hyperthermia combined with ionizing radiation (radiotherapy). The electric field and the radiofrequency current produced both the thermal and nonthermal processes. The thermal effects produce the elevated temperature as a thermal background to optimize the nonthermal impacts. The low frequency amplitude modulation ensures accurate targeting and promotes immunogenic cell death to develop the tumor specific memory T cells disrupting the malignant cells by immune surveillance. This process (abscopal effect) works like a vaccination. The low frequency amplitude modulation is combined in the new method with the high power pulses for short time, increasing the tumor distortion ability of the electric field. The new modulation combination has much deeper penetration triplicating the active thickness of the effective treatment. The short pulse absorption increases the safety and decreases the thermal toxicity of the treatment, making the treatment safer. The increased power allows for reduced treatment time with the prescribed dose.

KEYWORDS

Tumor, Pulsed Modulation, Nonthermal Excitation, Thermal Homeostasis, Cell-Killing, RF Current, Immunogenic Cell Death, [Apoptosis.com/oncothermia-journal/volume-37/](https://apoptosis.com/oncothermia-journal/volume-37/)

1. INTRODUCTION

Hyperthermia as a treatment for cancer has been recognized since ancient medicine. Conventional hyperthermia aims to achieve uniform heating of the tumor mass. The desired uniform heating condition forms the basis for dose calculation in clinical practice. Heat has been continuously employed in oncology, expecting to narrow the therapeutic gap caused by cellular distortion efficacy limitations and conventional treatments' toxicity (see Figure 1). Electromagnetic heating accelerated the application of oncologic hyperthermia, making it a significant tool in the oncotherapeutic arsenal. While electromagnetic heating revitalized hyperthermia methodologies, the underlying principle remained unchanged: exposing the tumor to high temperatures. This heat, coupled with the heightened metabolic demands of malignancies, leads to ATP depletion [1], severe hypoxia, and, ultimately, acidosis, which contributes to cancer cell death.

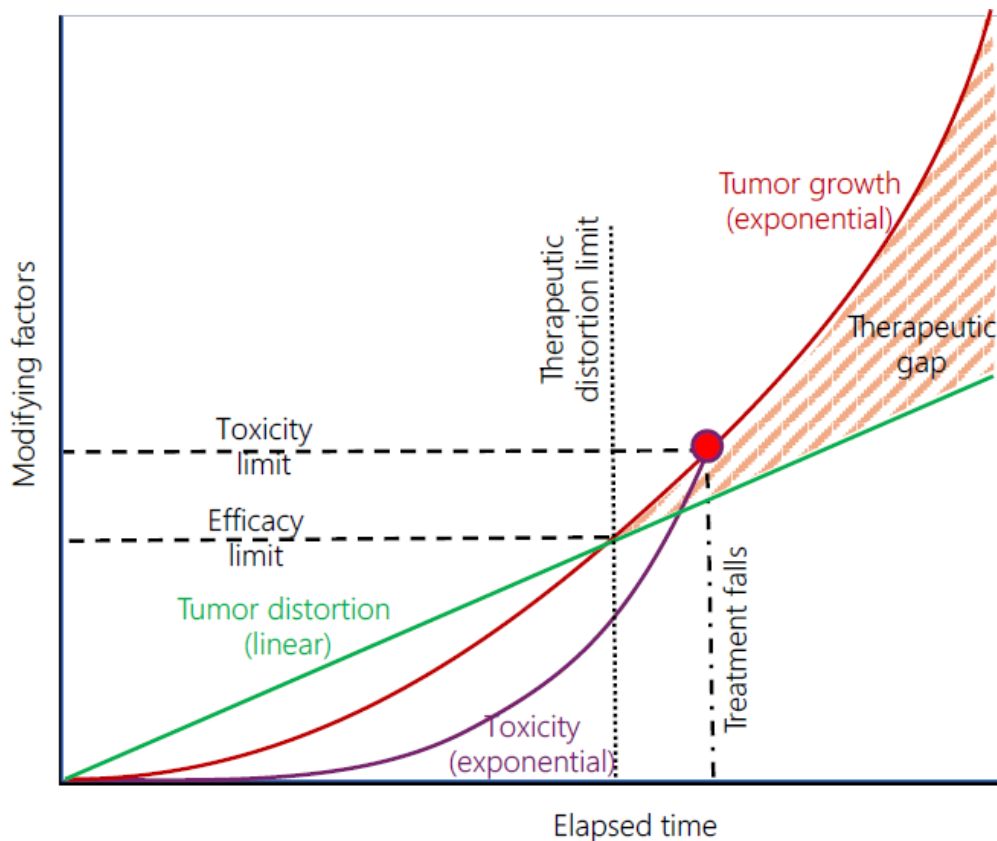


Figure 1. The development of tumors and their distortion. Cancer grows exponentially over time, while the distortion rate is linear. The exponential growth exceeds the distortion rate if the distortion cannot eliminate the tumor. Another limitation factor is toxicity, which is usually polynomial by time. The therapeutic gap is the missing therapy efficacy to the intensive growth of tumors.

The simple heating concept, however, does not consider thermal regulation and general homeostatic control of the body. The regulation mechanisms try to compensate for the unusual changes in the target and repair the damage caused. The stress proteins give a molecular basis of cellular protection [2], and numerous enzymatic reactions are activated to correct the unbalanced processes [3]. The primary regulating tool is the bloodstream [4], which tries to recool the heated volume, and the subcutaneous capillary bed works like an active heat exchanger to ensure the cooling capacity [5]. Oncologic hyperthermia can be a complementary tool to conventional therapies to reduce the therapeutic gap, increase the efficacy of traditional treatments, and expand patient survival with an acceptable quality of life. The increased blood flow can be used for reoxygenation to block the radiation- damaged DNA repair by enzymes, and it can also be used to deliver more drugs to the tumor and increase their reaction rate by elevated temperatures. However, the therapy is limited by precise targeting (the heat spreads), thermal toxicity (burning in the healthy tissues), and the risk of malignancy by bloodstream- assisted malignant dissemination. Due to the complications, the heating task has a complex multilevel approach. The technical solutions must fit the biological and technical requirements and be optimal regarding quality assurance and medical regulations (Table 1).

Table 1. The biological, clinical, and technical tasks address different therapy requests, which must be optimized.

Tasks		
Biological	Clinical	Technical
Tumor cell destruction	Tolerable temperature	Appropriate energy delivery
Tumor-selective cell destruction	Destroy the malignance	Appropriate focus and tuning of the energy
Immunogenic cell death (ICD)	Antitumor immune-effects	Do not destroy the malignant info from the dying tumor cells
Complementary possibilities to conventional therapies	Improve the therapeutic effect, decrease the therapeutic gap	Make appropriate blood flow regulation
Kill the malignancy independently from its depth	Give also proper dose to deep-seated tumors	Increase the effective penetration depth of energy delivery

2. THE CHALLENGES OF ONCOLOGICAL HYPERTHERMIA

Hyperthermia with electromagnetic energy delivery faces multiple challenges.

- 1) Due to the regulation of thermal homeostasis, we quickly lose control of heat localization inside the body. The temperature is automatically spread in the neighborhood of the target. The growing temperature intensifies the thermal homeostatic regulation and tries to cool the heated volume primarily with increased blood flow.
- 2) In long-term treatment, heat diffusion and gradually heated blood heat the whole body to the fever range. This elevation could help the development of micrometastases due to the increased metabolic reaction rate. At the same time, due to the homeostatic regulation compensating for the increasing temperature, the metabolism decreases in the healthy parts.
- 3) The more extended thermal equilibrium (keeping the tumor at a fixed temperature) replaces only the heat loss primarily caused by the blood thermal exchange. This period has much less cell destruction than the heating phase when the SAR intensively forces the apoptotic processes [6].
- 4) Heat Shock Proteins (HSP) are intensively developed and play a key role in protecting the tumor against prolonged exposure to high temperatures. HSP requires 48 hours to return to the baseline [2].
- 5) The long thermal equilibrium helps the dissemination of cancer cells from the tumor, mainly because of the high blood flow gradient at the tumor boundary, which has a high proliferation rate [7]. It increases the risk of metastases, drastically reducing the patient's survival time and quality of life.
- 6) The high blood flow may support tumor growth and increase the risk of dissemination. To reduce the blood flow, we have to limit the average temperature, while for the effectual cell distortion, we need higher than the physiological limit ($>42^{\circ}\text{C}$).

- 7) Growing hypoxia with more prolonged heating may increase the necrotic core of large tumors, which increases the risk of tumor lysis or cytokine storm. The goal is the apoptotic cell destruction of cancer, which needs to trigger proper signals and lower destructive heat.
- 8) The further complication is to choose a power high enough for the excitation but not too high to cause necrosis and avoid thermal toxicity, primarily the burning of the skin and the fatty tissues.
- 9) The electric field drives the energy absorption. The tumor is targeted by radiative, capacitive, or inductive coupling, which puts the energy into the tumor. Technically, the process is like the antenna matching at the radio broadcasts. However, the antenna has fixed parameters that do not change the coupling conditions. The electric parameters of the human body vary by patient and tumor location during the treatment, and the tumor's electric properties may change during the treatment, so the coupling must follow the changes.
- 10) From the immunogenic point of view, the average tumor temperature must remain under 40°C. Higher temperature arrests immune activity [8]. The growing systemic temperature could give extra temperature to the heated tumor and go over the immunogenic limit, decreasing the mEHT efficacy.
- 11) The penetration of energy absorption sharply declines from the surface to depth into the body.

3. METHOD

To solve the above challenges, a novel method, the modulated electrohyperthermia (mEHT, oncothermia®), was developed [9]. The isothermal heating concept is unrealistic due to the massive thermal and bioelectromagnetic heterogeneity, and the energy absorption fits the structural peculiarities of the living organism. The technical solution of optimal energy absorption must consider the complexly interconnected micro and macro heterogeneities and be compatible with the physiologic parameters regulated by the organism's homeostatic control. The modulated electrohyperthermia (mEHT) changed the hyperthermia paradigm, using heterogenic, selective heating to target the death receptors of the malignant cells and induce apoptotic signals to destroy these cells [7]. In heterogenic heating, the specific absorption rate (SAR) changes by the position of the absorber (x) and time (t), so the complete energy is the sum of $SAR(x, t)$. The absorbed SAR is used to raise the temperature of the target by heating its environment by heat conduction and bloodstream-assisted heat convection. The point heating enlarges over time and heats the mass by the contained energy-absorbing molecules (Figure 2).

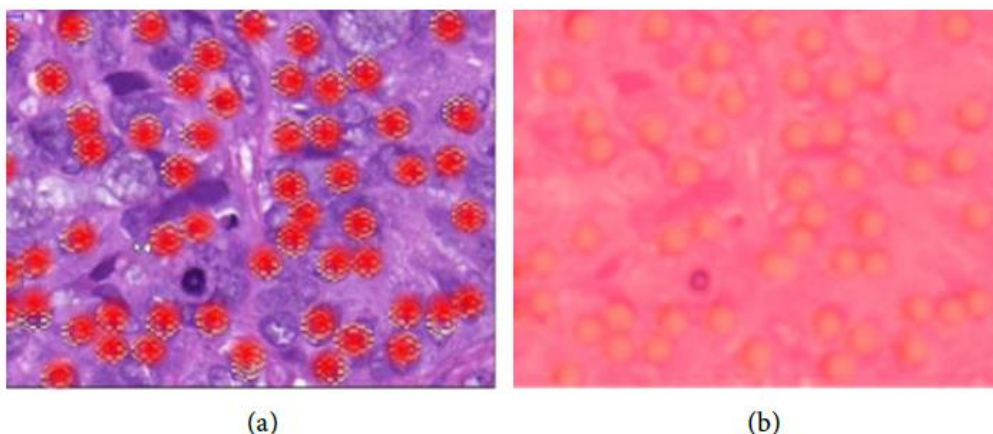


Figure 2. mEHT heats heterogeneously in selected molecular clusters and transmembrane proteins (membrane rafts) on malignant cells. (a) The molecules' energy absorption heats at high temperatures (yellow dots), which conductively heats the cell (red circles), and the induced blood flow heats the complete mass convectively; (b) When the power is off, the mass starts to cool primarily by the blood flow, and in the end, it will be almost isothermal at a lower temperature.

The method uses amplitude-modulated 13.56 MHz carrier frequency [10] [11] to deliver the appropriate energy for hyperthermia processes. The key to proper focusing is the use of the electric and thermal heterogeneity of the tumor. The tumor specialties differ from the healthy tissues, and those allow selective heating of the cancer cells. The high metabolic rate of malignancy and the missing healthy cellular network can conduct the appropriately chosen radiofrequency (RF) current differently.

The cytoplasmic transmembrane proteins of malignant cells selectively absorb the RF current delivered energy [12]. The mEHT method selectively targets the membrane rafts of the malignant cells [13]. The malignant cells have a denser lipid-raft population on their membranes than their healthy counterparts [14] due to the high density of the unbounded transmembrane proteins from the broken intercellular network. The targeted energy absorption makes the precise and well-focused hyperthermic impact possible (Figure 3).

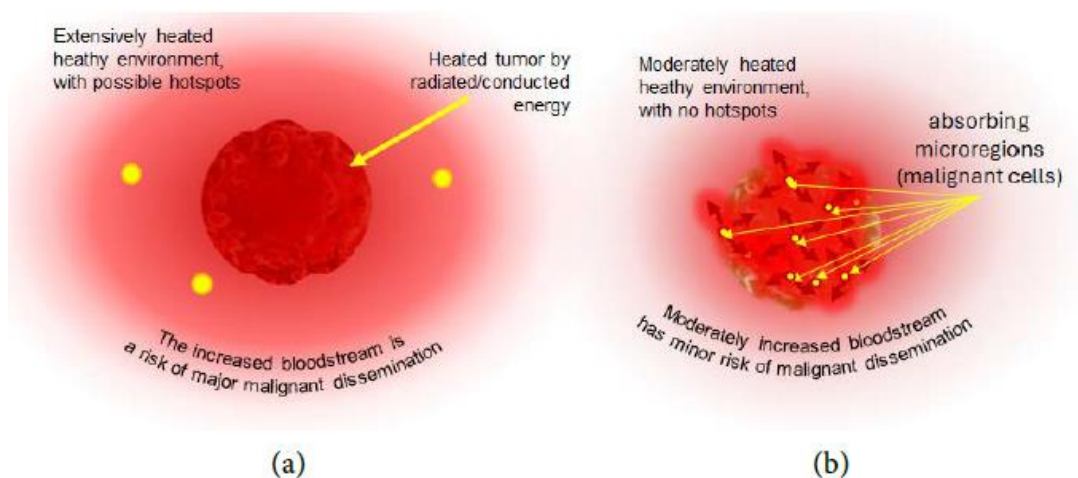


Figure 3. The difference between conventional and heterogeneous heating. (a) The conventional hyperthermia aims to heat the tumor isothermally, homogeneously heating the tumor mass; (b) The naturally selected absorbers (clusters of transmembrane proteins, membrane rafts) heat heterogeneously, focusing the malignant cells only.

The thermal effect is proportional to the radius of the targeted rafts [15]. The weight heating of the nanoparticles is approximately 100 – 1500 kW/kg [16]. The thermal effect of mEHT acts not only on the rafts but also heats the TME and the neighboring tissues to a less significant level. Thermal energy absorption of the rafts by mEHT is like the nanoparticles, and it is approximately 350 kW/kg, which is comparable to nanoparticle heating [11] (Figure 4). The energy absorption process is more complex in the case of rafts than in nanoparticles, having additional molecular excitations [14] and nonthermal processes that do not happen in nanoparticle heating. The nonthermal impact excites protein complexes, which thermal processes heat to an optimal chemical reaction rate. The

thermal influence of this heterogenic heating is identical to conventional hyperthermia, but it targets, more precisely, the malignant cells.

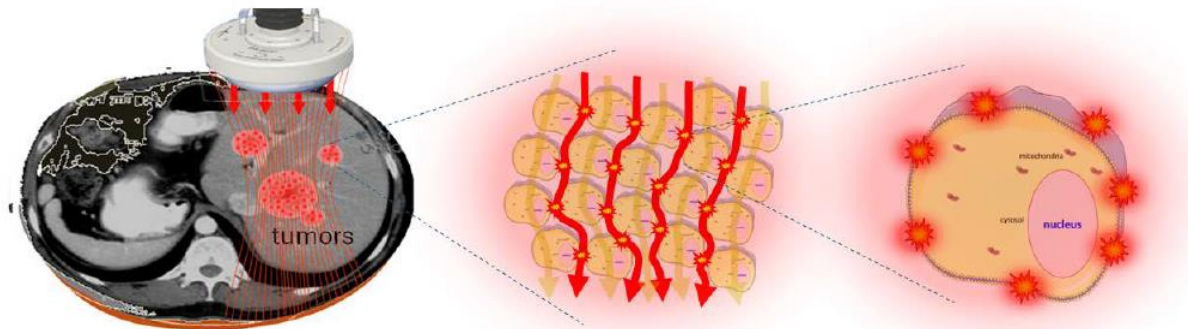


Figure 4. The applied special RF current chokes the tumors. The majority of the current flows in the extracellular matrix, and excites the transmembrane proteins (rafts). The absorbed energy on rafts heats the cells and also triggers intracellular signals.

The rafts are heated to optimally high, but the tumor temperature remains under 40°C. This relatively low average temperature avoids downregulating the cytotoxicity of immune impacts, which are inhibited in higher temperatures [8] [17] [18]. However, on the contrary, conventional hyperthermia intends to have direct cellular thermal damage, which needs higher temperatures (above 41°C – 42°C [19]). The heterogenic, selective heating matches both seemingly contradictory demands, having high temperatures (>40°C) in nanoscopic absorbers, and the tumor mass average remains under 40°C.

The mild average temperature (39°C – 41°C) intensifies blood perfusion [20], optimizing the reoxygenation for complementary radiotherapy and delivering chemo drugs in a targeted manner, enhancing their reaction rate. The preclinical experiences verify [21]–[23], and the clinical studies validate these effects [24] [25].

The delivered thermal effect is concomitantly applied with the nonthermal processes offered by the electric field (Figure 5). The heat drives the chemical reaction rate, and the thermal energy activates numerous temperature dependent molecular pathways. The nonthermal energy promotes such processes which are normally not excited by heat only, similar to the chemical “machinery” of the life, which needs different energy involvement than the heat energy alone. The synergy of the thermal and nonthermal energy absorption produces apoptosis of the malignant cells.

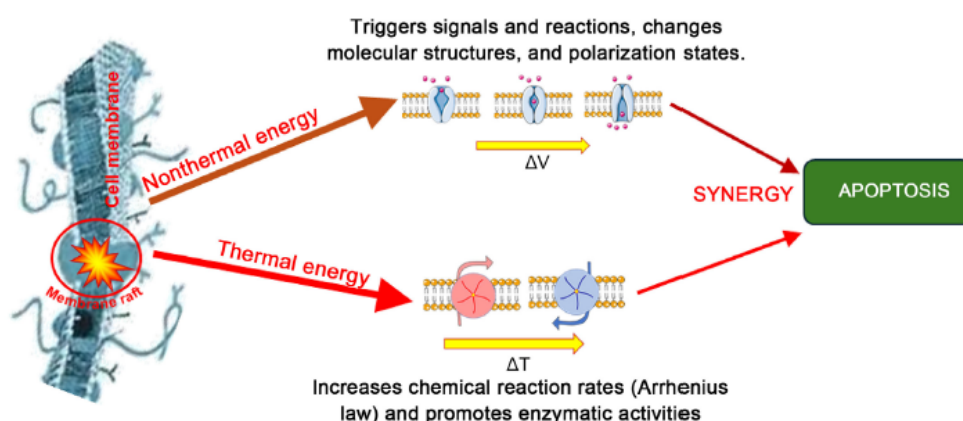


Figure 5. The synergy of thermal and nonthermal energy produces apoptosis of malignant cells.

The technical challenge of optimal and variable coupling is solved with precise and dynamic impedance-matching [26] [27]. The electronic solution is tuning the system to the measured impedance of the treated individual. The tuning seeks to form a touching situation as a purely metallic electrode would be fitted to the skin directly. This matching situation calculates the actual energy loss carefully, controls the reflected power, and matches the resonant compensation of the surface capacitor of the adipose tissue. The impedance-matched capacitive coupling produces the appropriate RF current density. The matching of the load in the RF circuit harmonizes the multiple interconnected parameters responsible for the conductivity [28]. The matching transforms the varying complex impedances at constant carrier frequency 13.56 MHz to nominal reference (conventionally real. 50 Ω) impedance [29]. The selection of the membrane rafts for energy absorption solves this challenge [13].

Our goal is to apply the pulsing technique for the mEHT method. The pulsing could be characterized by the duty cycle (D), which is the ratio of the pulse width (t_p) and the repetition time (t_r). This D time ratio is equal to the ratio of the mean power (P_m) and the pulse power (P_p):

$$\frac{t_p}{t_r} = \frac{P_m}{P_p} = D \quad (1)$$

The novel pulsing modulation and the standard modulation of mEHT [30] [31] give a unique mixture (Figure 6).

4. RESULTS

The solution to optimize the ratio of the thermal and nonthermal components of the RF current needs a precise fit to the individual case, which changes in every treatment. The active impedance-guided capacitive solution (like mEHT) can use the bioelectromagnetic specialties of the malignant cells directly by RF current flow when it is matched to the optimized current. The apparent contradictory requirements (high frequency for selection and low frequency for action) are solved with low-frequency modulation of the high-frequency carrier [31] [32]. With the high carrier frequency, the interfacial polarization effect [32] is optimal for the selection using β/δ frequency dispersion range [32] [33]. However, the optimal excitation, the polarization-dependent extrinsic molecular signal of selected membrane rafts, requires low frequency, which is in the α -dispersion range. The α -dispersion frequency in living tissues refers to the frequency range where the dielectric properties of the tissue exhibit significant changes due to the relaxation times of molecules and ions present in the tissue [34]. The cell membrane rectifies the modulated signal [35]–[37], cutting the high-frequency carrier and presenting a simple low-frequency spectrum of the modulation signal alone.

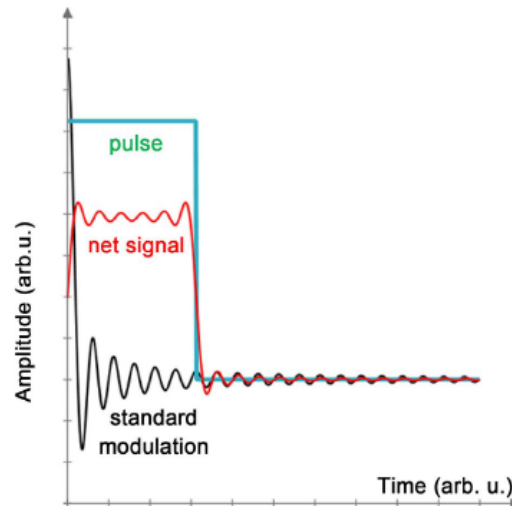


Figure 6. The modulation and temperature during a limited time pulse. The net signal of the modulation is the convolution of the mEHT standard and the pulsing. When no next pulse happens, the temperature returns to the baseline.

The precise impedance matching, adapted to all target changes, promotes a high penetration depth into the body [38]. The tuning adaptation constrains the spreading, but the depth does not exceed 20 cm [39].

The current density (j) is a characteristic dose in impedance-matched situations like mEHT. It does not depend on the technical losses outside of the target. When the matching is correct, the current that goes into the body in one electrode comes out in the other without loss. However, isodose of j is only an average in heterogeneous material. The statistical average of the current density over the whole target is similar to the dose in chemotherapy, where the drug distribution is also heterogeneous in the various tissues. Still, in the statistical average, we declare homogeneous drug delivery all over the body, giving the dose by the subject's size.

The electric field (E) generates the j :
$$j = \sigma E \left(\frac{A}{m^2} \right),$$
 where σ is the average conductivity of the tissues where the current flows through.

The electric field drives both the thermal and nonthermal processes. Consequently, keeping the current density constant provides the dose for the treatment. The j in first approximation does not depend on the A size of the electrodes. The $A = r^2\pi$ size of a circular electrode with a radius r defines

the j current density from the provided current (I):
$$j = \frac{I}{A}.$$

According to the Ohm-law, the electrode voltage (V) and the resistivity (R) between the electrodes

determines the I :
$$I = \frac{V}{R}.$$

In a first approximation, the current flows in a cylinder between the two circular electrodes, so

$$R = \frac{d}{\sigma A},$$

where d is the distance between the electrodes, so the thickness of the body.

Consequently, $j = \frac{V\sigma}{d}$, constant when the applied voltage does not change on the same patient. The power (P) of the absorbed energy depends on the square of the field:

$$P = \sigma E^2 = \frac{1}{\sigma} j^2 \left(\frac{\text{W}}{\text{kg}} \right).$$

When the power kept constant, the $j = r \cdot \sqrt{\frac{P \cdot \sigma \cdot \pi}{d}}$, so the current density increases by the electrode size when the power is constant. This surprising increase of j is approximately compensated with the growing radiation loss by the electrode size, so the simple dose monitoring by the power [39] [40] could be the dosing instead of by the local temperature high efficacy of current matching [41]. The higher power increases the current density dosing by $j \sim \sqrt{P}$.

Conventional hyperthermia heats the tumor mass and needs much higher incident power than mEHT, which heats only a tiny fraction of the membrane rafts of the selected malignant cells.

Conventional clinical hyperthermia uses intensive surface cooling to avoid skin and subcutaneous burns caused by heating. The cooling takes away a part of the incident power, which has to be higher to provide the same effect, so the treatment current density increases by $j \sim \sqrt{P}$.

Due to the thermal mass heating, the complete penetrating power heats the target. However, the situation differs in mEHT practice. A high current is also desired in mEHT, but due to the precise dosing, the cooling of the surface has to be somewhat limited. The consequence of the relatively low power, which is optimal for clinical control, is that it does not allow for an increase in the current density. It was shown in vitro mEHT experiments that when the average thermal component is arrested by the cooling down of the cells back to the 36°C baseline, the apoptosis grows by the incident power, which determines the RF current density. However, the applied cooling (energy loss) significantly modifies the incident power so that it cannot be used for dosing. The dose is the current which is forced to flow through the patient. The current flows through the patient independently from the energy loss, which characterizes only the absorbed SAR. The electrode size distributes the current, but the j current density does not depend on it when the voltage is kept constant.

The current density represents an isodose in the mEHT. The nonionizing RF radiation works like the isodose intensity of ionizing radiation in radiotherapy. From a hyperthermia point of view, it is a complementary therapy of nonthermal nonionizing radiation to conventional thermal treatment. The mEHT thermally generates optimal conditions for the concomitantly present nonthermal electric field. The combination improves the reaction rates and enzymatic reactions.

Thermal and nonthermal effects synergy is like conventional hyperthermia with radiotherapy [42]. The modulated RF signal of mEHT triggers resonant excitations of the proteins [43], driving an extrinsic signal pathway for apoptosis [44] in a dose-dependent way [6].

The rise in temperature has a typical curve in hyperthermia (Figure 7). The starting period is the same as for any other solid state; the temperature linearly grows by the constant power. The linear slope declines when the target starts to exchange heat with the intensified blood flow and surroundings. The temperature grows slower despite the same power and becomes steady when equilibrium with the cooling effects. In this period, the absorbed energy equals the heat loss, replacing only the energy exchanged with the environment. Cellular damage is controlled by the ratio of the heating temperature to the growing and keeping periods. The heating period when the temperature grows has essential importance. The increasing thermal effect and the intensive molecular excitations (primarily by polarization nonthermal effect) characterize the process due to the thermal improvement of the molecular reaction rates. The thermal and nonthermal factors are unchanged in the heating phase of a stable temperature. However, it is observed that mEHT significantly increases the apoptosis in the heating-up period, while the temperature-keeping equilibrium has only moderate cell destruction [6]. Applied step-up heating uses this difference to improve apoptotic processes [45].

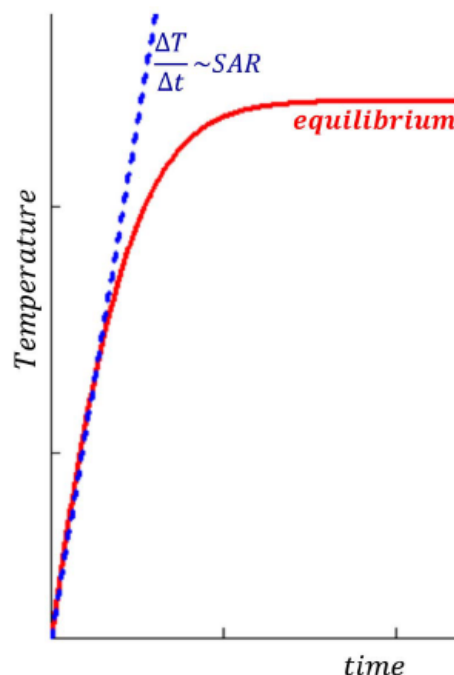


Figure 7. Typical pattern of temperature development. The initial linear growth heats the tumor, while the heat transfer to its neighborhood modifies it after a time lag. The formed equilibrium needs only such energy, which replaces the heat by exchanges, while the tumor remains on constant temperature.

In equilibrium, the absorbed heat replaces the lost energy, not heat further at higher temperatures. Equilibrium depends on the heat loss to the environment of the tumor. Primarily, the blood flow regulates the loss of energy [46] and secondary the heat conductivity of the host tissues, which is driven by the temperature gradient between the tumor and the host. Usually, the SAR for

equilibrium is less than that for temperature-grooving tumor heating (Figure 8). The timing of the decline of the initial linear slope depends on thermal conditions and in vivo on the physiologic feedback, which starts to cool the heated volume. The feedback differs by the tumor size, tumor location and the personal reaction abilities in treatments and of course, defined by the temperature limiting preset conditions (usually $\leq 42^{\circ}\text{C}$) where the treatment continuously runs.

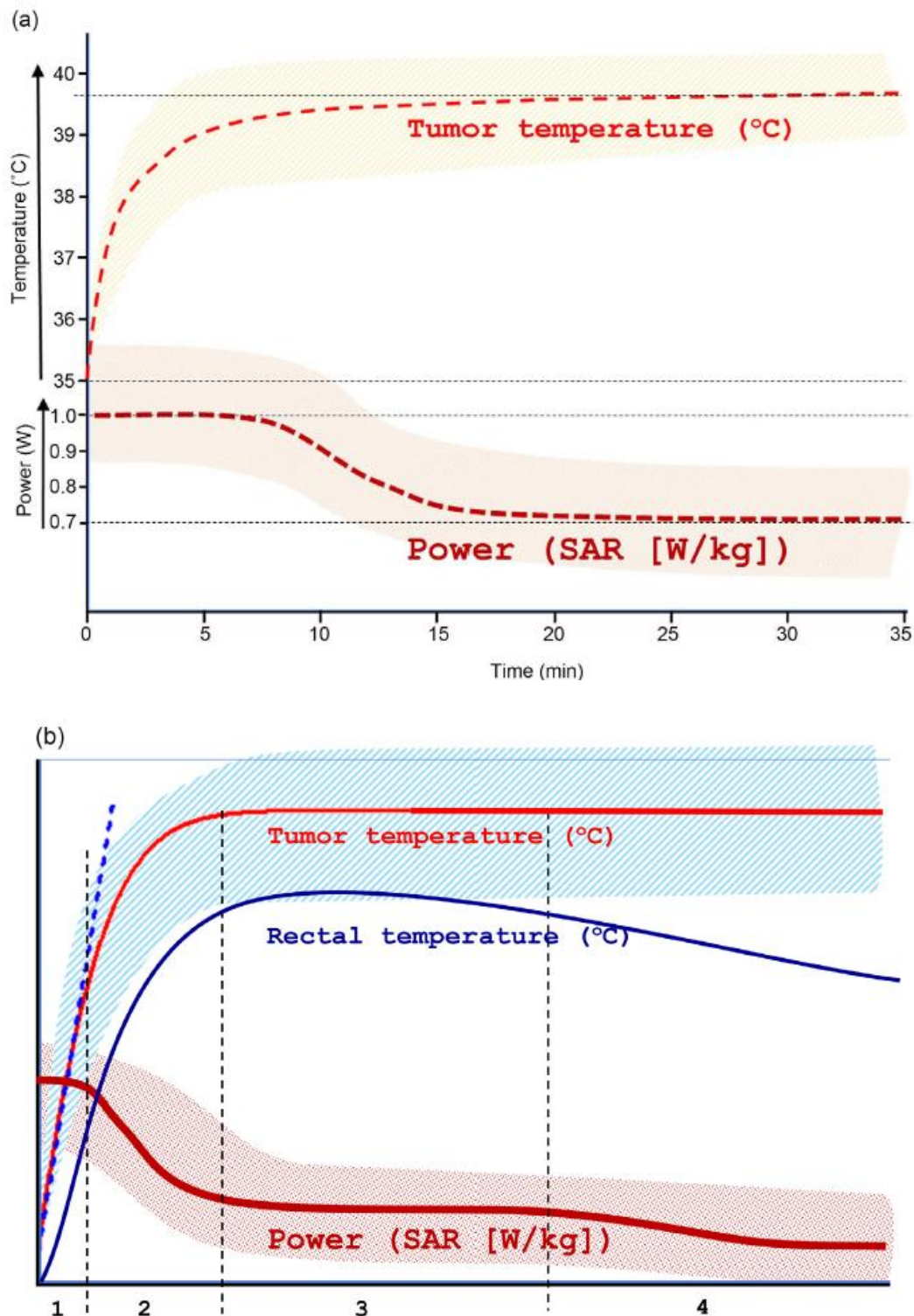


Figure 8. Relation of power and temperature patterns by time. The requested power is higher at the heating phase for temperature increase than in the phase of heat exchange equilibrium. (a)

Both the power and the temperature are unchanged in the equilibrium (such shape is shown in vitro [47]); (b) While the tumor temperature is in equilibrium the power may be decreased in pulmonary heating, when the intensive breathing could not stabilize the heat-exchange but the final equilibrium needs less energy. (Such shape is shown in vivo pulmonary treatment [48].

When the power is switched off, the target is cooled by the bloodstream and by heat conduction to neighboring tissues. The physiological feedback of homeostasis has a time lag of a few minutes. Until this time, the rafts and cells are heated quasi-adiabatically without heat loss by the effectively cooling bloodstream. During this period, the temperature (T) rise solely depends on the SAR only,

$$\frac{\Delta T}{\Delta t}$$

allowing the measurement of the effective SAR with the $\frac{\Delta T}{\Delta t}$. Afterward, the absorbed energy does not increase the temperature. The temperature development has a saturated curve. The physiological self-time in humans, which determines the time lag of the heating modification, is ~6 min [46] (Figure 9). The temperature change follows the thermal washout. The original application of the washout time was to characterize the drug washout [49] (like chemo-drugs, radiofarmacons, tracers, blood-delivered molecules, and nanoparticles) from the tissues, but in general, it is also used for the “wash out” of excess heat energy from the target. The primary difference between the thermal washout and the washout of various blood-delivered particles or molecules is the heat diffusion, which depends on the tumor and the host thermal properties, depends on the blood flow in the tumor and its neighborhoods, and depends on the temperature difference (gradient) between the heated tumor and its host tissue. The metabolic heat also affects the thermal washout.

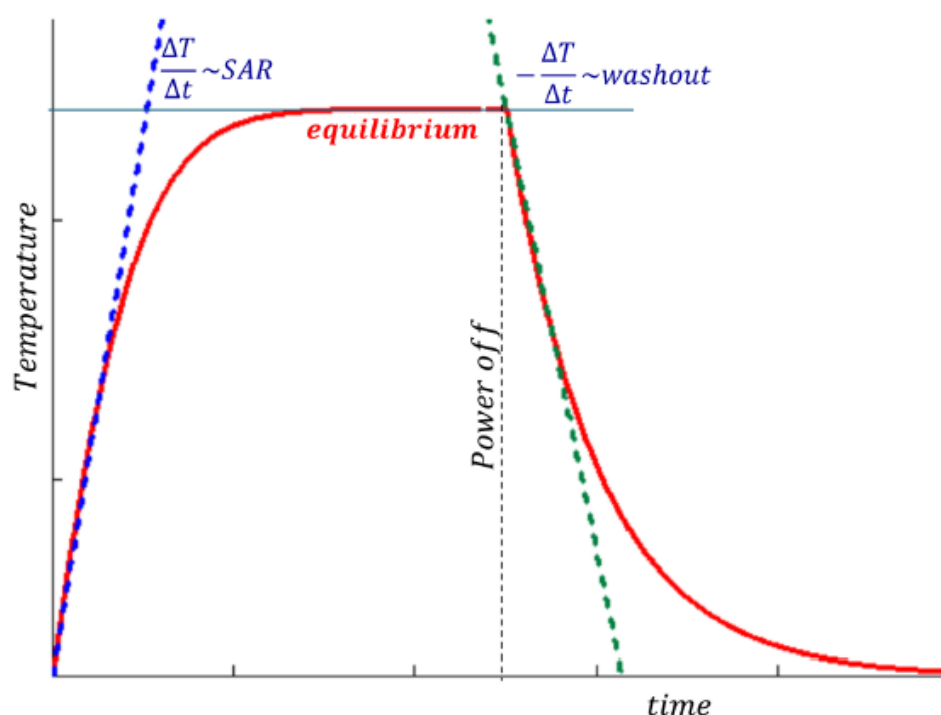


Figure 9. When power is off, the wash out by blood flow intensively cools down the tumor to the baseline.

The living system has a self-time which depends on the individual complexity [50]. Thermodynamic optimizing varies the self-time [51], which is connected to allometry structure [52],

$$\alpha \approx \frac{3}{4}$$

and it scales with the allometric factor [53]. The time of thermal washout can be modified by changing the metabolic rate by lowering the temperature, and it will cause a longer tail to the washout function in time. Consequently, metabolic modification is an addition to the dynamism of cooling. The physiological self-time of the thermal washout is ~6 min in healthy condition. For example, the thermal washout of a tissue from 42°C after 30 – 60 min microwave heating is ~6.5 min [54].

The apoptotic cellular degradation could be used for dosing in the active heating period [6], and it shows the efficacy of the therapy in vitro (U937 cell line) [55]. The current is proportional to the percentage of apoptosis [56], because the necrosis remains low (<2%). The standard mEHT treatment has a synergy of thermal (41°C) and nonthermal effects at 8W without cooling energy loss, while the samples that were forced to remain in the temperature baseline produced only nonthermal effects depending on the applied power [56] (Figure 10).

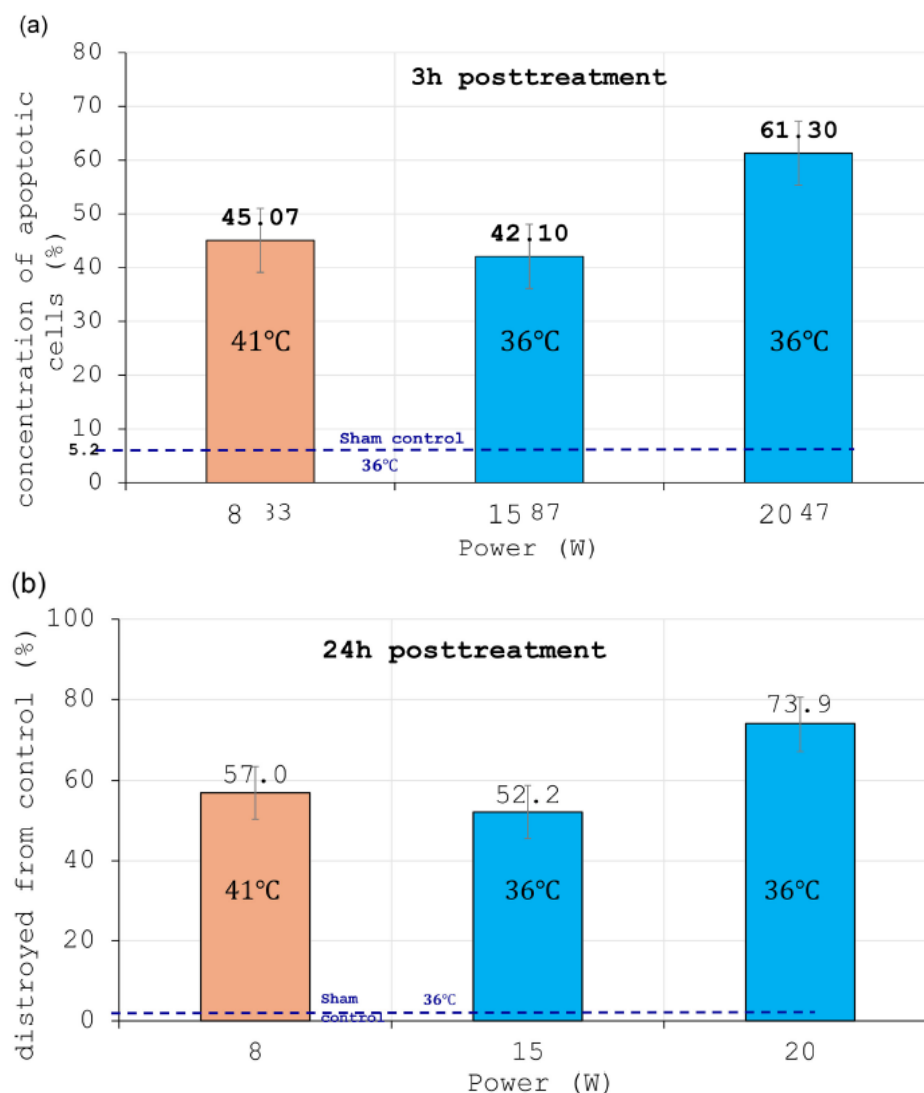
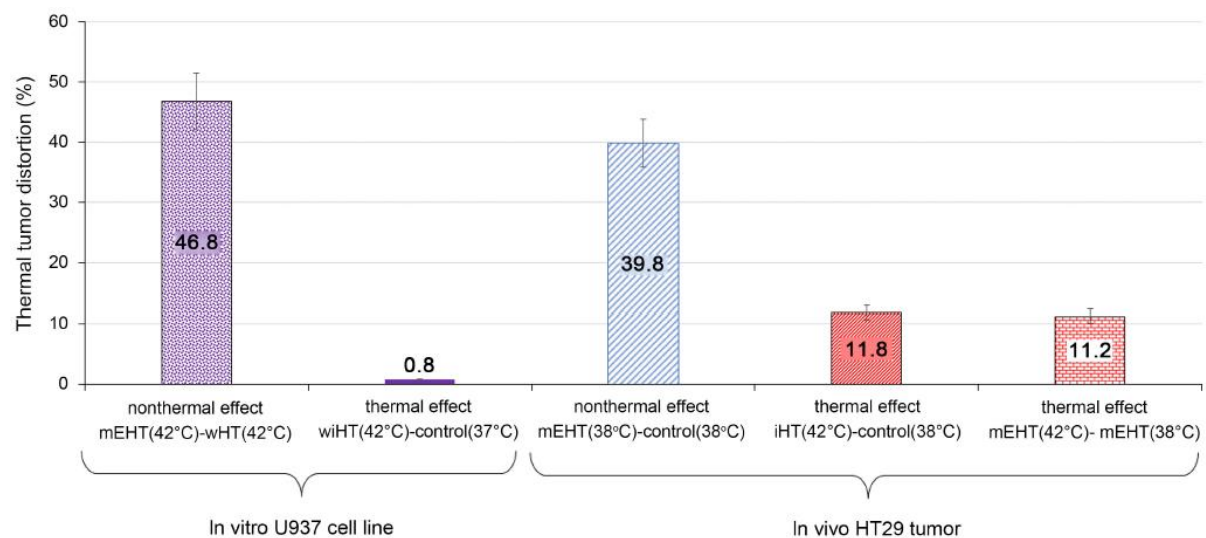


Figure 10. The cellular distortion of mEHT in vitro U937 cell line [57]. Intensive cooling of the sample kept the cell-contained medium at 36°C, while the standard treatment was at 41°C. Due to the energy taken by cooling, higher power was requested to maintain thermal balance. (a) 3 h after treatment the standard mEHT shows apoptosis (necrotic cells < 2%), with various power (the applied current is $I \sim \sqrt{P}$), and temperatures. The cooling forced samples remain in the temperature baseline, so show the impact of the nonthermal processes only. (b) 24 h posttreatment results with same conditions as (a).

The in vivo experiments on HT29 tumors in xenograft mouse model also proves the difference between the thermal and nonthermal components of the incident power [57] (Figure 11).



The heating pattern does not follow the rectangular pulses. The spreading of the heat extends the temperature growth to the neighboring volumes. The result depends on the pulse width and the heating pattern of the target (Figure 12).

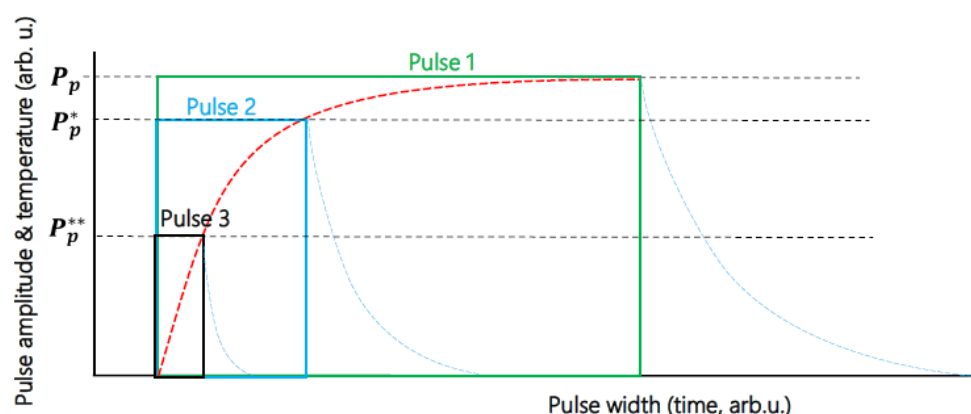


Figure 12. Various pulses have different temperature patterns. The short pulse is active in the linear part of the temperature growth (pulse 3), and quickly cools back to the baseline from this little temperature increase. The wide pulse contains the equilibrium phase (pulse 1), while in the middle range the pulse cuts the temperature curve in indefinite position (pulse 2).

The temperature of the tumor depends on the target's thermal parameters (Figure 13).

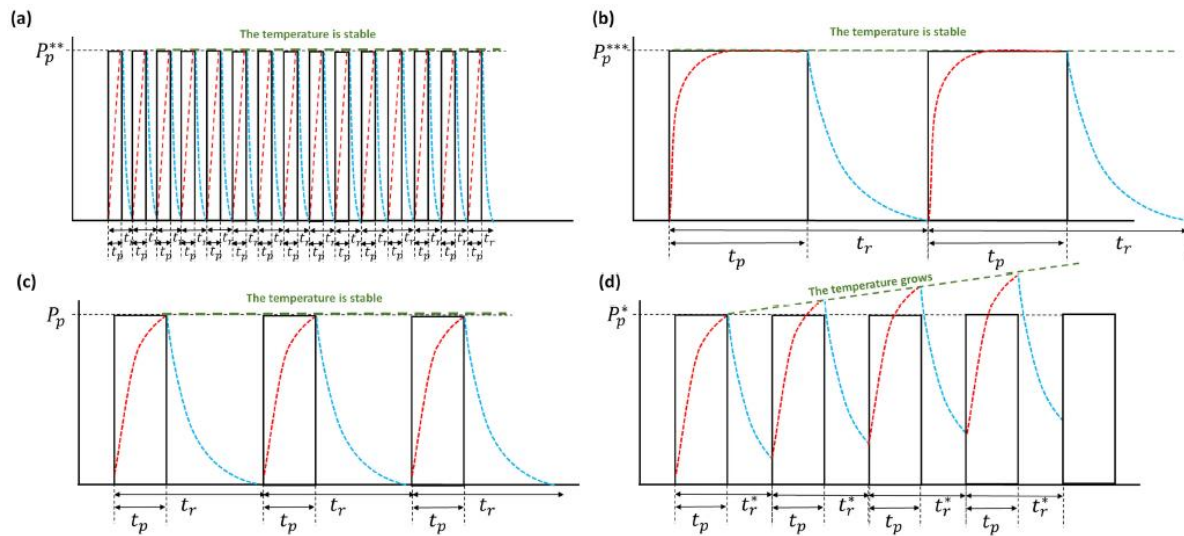


Figure 13. The electric pulsing and its thermal reaction differ and depend on the duty cycle

$$D = \frac{t_p}{t_r} = \frac{P_m}{P_p}$$

(a) The pulse heats the material only in its starting (quasi-adiabatic) period when the physiological feedback is not active (pulse 3 in Figure 9), and the break period of the pulsing is long enough to cool the energy-absorbing microdomains back to baseline from the relatively small temperature growth. The temperature is practically unchanged, but the nonthermal effects could be activated when their characteristic time is shorter than the t_p pulse width, despite the possible high repetition frequency. (b) When the pulse is long enough till the stable temperature (pulse 1 in Figure 9), and the break time is enough to cool the target; the temperature remains in the saturated equilibrium. (c) When the pulse length is in the transition phase of the temperature development (starting physiological feedback, pulse 2 in Figure 9), and the break is enough for cooling to the baseline, the temperature will be stable but will not reach the same value as the thermal equilibrium in (b). (d) When the pulse repetition is quicker than necessary to cool down to the baseline, the temperature rises, despite the constant P_p power. The change of t_0 may drastically change the thermal pulse at the same electric pulsing. The temperature growth can happen in all the pulsing in Figure 9, it depends on only the necessary time to return to baseline temperature.

The small duty cycle may keep the temperature at the baseline. Noteworthy, the micro absorbers experience growth and drop in temperature, while the average mass temperature is unchanged (Figures 13(a)-(c)). When the t_r repetition period is longer than completing the physiological feedback (about 6 min), then the continuous and pulsed heating do not differ in the measured temperature. However, at larger duty cycles, the cooling period is not enough to reach the baseline again, and the temperature of the mass average grows repeatedly (Figure 13(d)).

Learning that the heating phase has the highest SAR (see Figure 5) in vitro experiment showed that higher SAR produces higher apoptosis [6]. Using this observation a considerable increase of apoptosis was shown in pulsing experiments in vitro [6] and in vivo [58]. Another in vivo experiment strongly supports the pulsing idea [59] (Figure 14). The experiments show the importance of the pulse amplitude, which has to be high enough producing better efficacy than the continuous mEHT.

Power determines the response. When the pulse's power is in the continuous power range, the response of pulsed mEHT does not differ from the standard mEHT efficacy. When the pulsed power is more than double than the continuous one, the pulsed treatment shows significantly increased response (Figure 15).

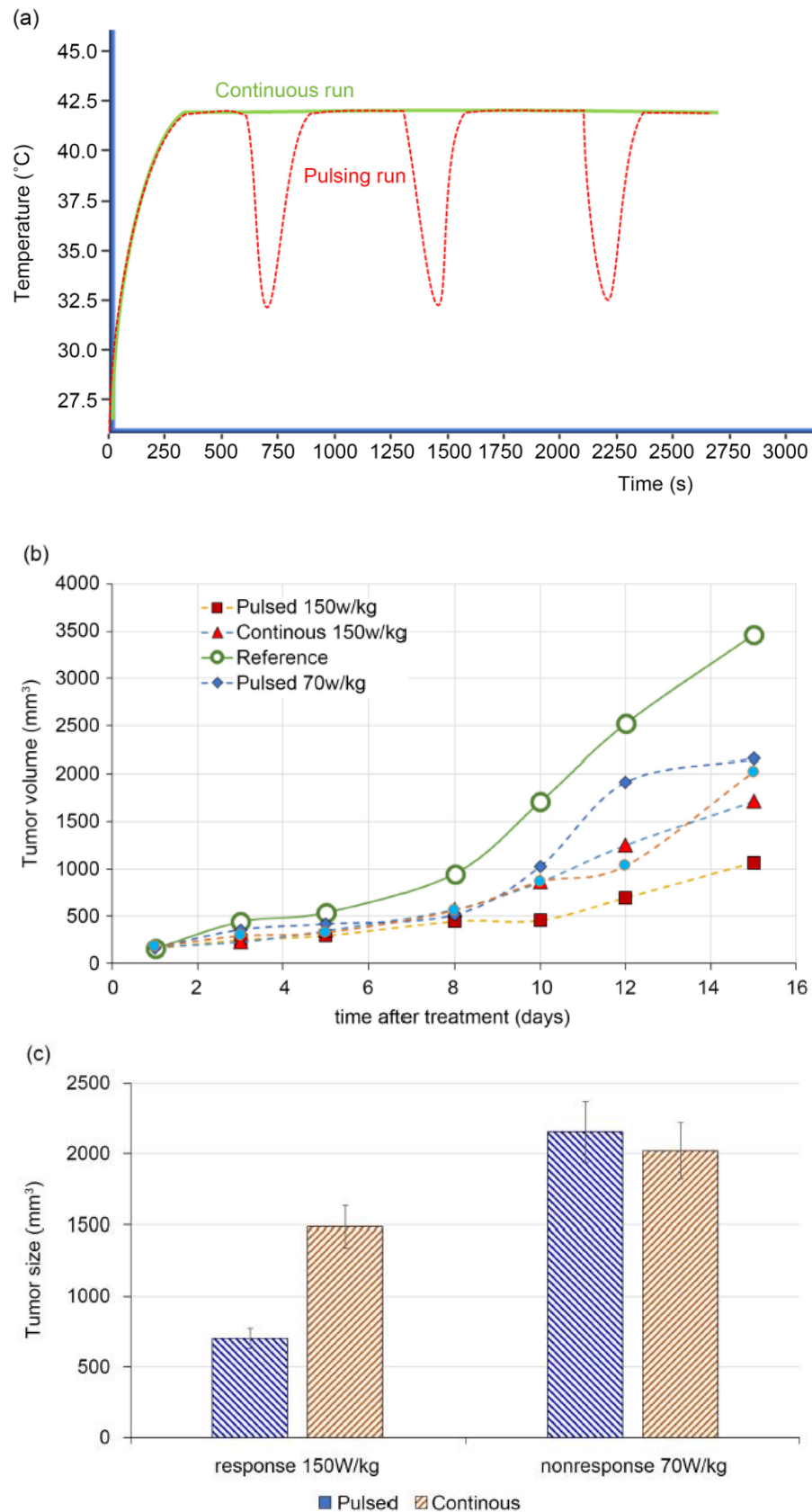


Figure 14. Pulsing in vivo experiment (HepG2 tumor in mice [60]). (a) temperature change in pulsed and in continuous mEHT treatment; (b) The tumor volume after treatment is continuous and pulsed treatment with standard SAR (70 W/kg) and high SAR (150 W/kg) measured by the initial slope of the temperature growth; (c) the results on the 16th day post-treatment.

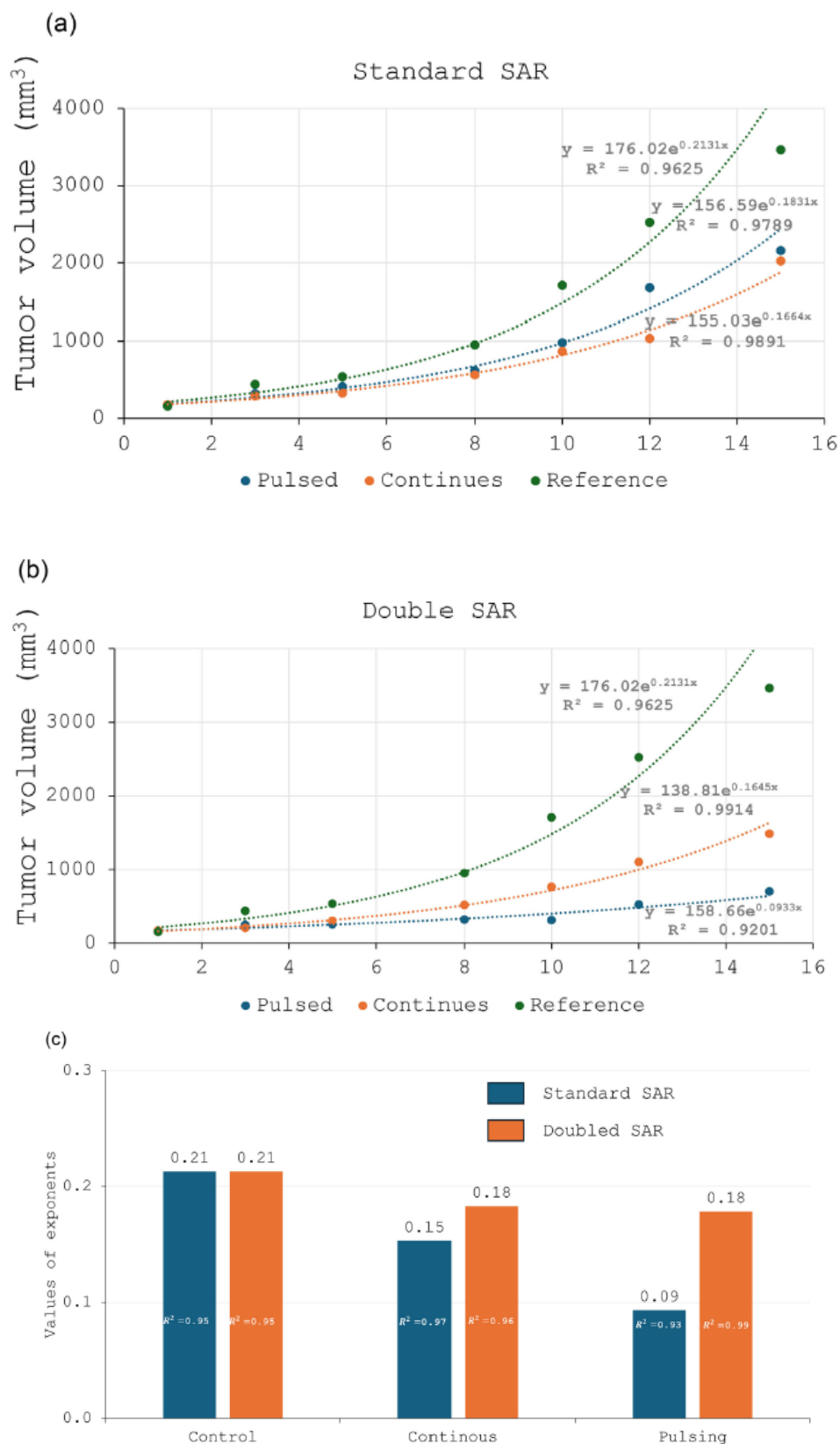


Figure 15. Comparison of the pulsing and continuous mEHT. (a) The continuous and the same SAR pulses do not differ significantly in tumor destruction results; (b) Increase the SAR by 2+, the pulsed efficacy has significantly better tumor destructive potential. The exponents and their correlation of the growing tumor after different mEHT treatments.

5. DISCUSSION

Different studies were performed to check the benefits of pulsing in hyperthermia applications. The theoretical estimation of the thermal effect of external heating with laser pulses in local thermal non-equilibrium approach [60]. The theoretical considerations in the bioheat model show that pulse heating is better for avoiding the thermal toxicity of healthy tissues [61]. Pulsing electromagnetic treatment is often used for electroporation, showing its better performance with higher pulse amplitude in combination with chemotherapy Bleomycin, which is shown in chemoresistant cancer stem cells in vitro [61]. The multicomponent complementary treatment (electro-radio-chemotherapy) also indicates the superiority of the pulsing application, studied in vivo (SCCVII tumor, C3H mice) [62]. The hyperthermia with magnetic pulses and high duty cycle (0.8 – 0.9) showed optimal results for breast cancer with minimized adverse effects [63] inducing damage to the tumor microcirculation. A low frequency, low amplitude alternating current makes electric stimulation, which massively and significantly enhances the efficacy of the complementary doxorubicin chemotherapy in numerous cell lines in vitro [64]. The pulsing targeting allows high temperatures in pulse with minimal loads of the healthy tissues around in malignant melanoma treatment [65]. All information in the pulsed hyperthermia approach emphasizes the method's benefits independently of the other energy source.

When the tumor temperature grows linearly in the initial heating period, the starting energy delivery creates noticeably different heating processes than when the temperature grows nonlinearly and slower. During the linear period, the target solely absorbs the energy, which is why, later, the different types of heat transports modify the temperature growth, and a part of the heat energy is lost for the heating of the tumor. The nonthermal effect also decreases in the nonlinear heating period because the current density decreases by spreading the RF current to wider areas by depth and time. The thermal optimization for nonthermal processes also decreases in the target. The pulsed electromagnetic energy shows a massive nonthermal effect when the pulsing power is high [66]. The experimental skin layers also may suffer pulsing heat damage [67]. The chemical and thermal process in pulsing may activate the TRPV3 vanillin receptor [68], which participates in immunogenic effects in mEHT applications [12]. The pulsed electric treatment induces transient reversible opening of the blood-brain barrier (BBB) [69]–[71], promoting drug delivery to the brain. The BBB opening mechanism could impact nonthermally, involving the tight junctions [72]. The pain management of the pulsed electric field is an active pain killer [73], which increases the quality of life of patients suffering from severe pain. Thermal cycling shows benefits in conventional hyperthermia, enhancing the anticancer processes shown in pancreatic malignant cells [74] and treating humans [75]. The oncological applications of pulsed electromagnetic sources have a prosperous future [76].

The pulsed hyperthermia applies heat in short and intensive absorption with controlled bursts with constant amplitude. While continuous hyperthermia has uncontrolled heat transfers to healthy surroundings, pulsed targeting is controllable and gives great advantages to precise therapy. It

reduces the risk of thermal toxicity in healthy tissues, promoted by the usually higher blood perfusion in the healthy tissues than in the tumors. Pulsed heating has a higher potential for tumor distraction due to the higher power maximum in the pulse, while the tolerability by patients increases due to the reduced overall heat coverage.

The new mEHT pulsing modulation has multiple additional benefits to the continuous mEHT operation. The RF current is an active factor of mEHT. The current selectively flows through the target. In pulsing conditions, the extreme power gives a proportionally sizeable current density, which makes an effect.

Pulsed heating can be more effective at killing cancer cells than continuous heating at the same average temperature. Pulsing during treatment starts a gradual temperature increase, which is crucial for inducing cell death and triggering immune responses. Increased blood flow to tumors between pulses delivers more oxygen and nutrients, enhancing heat's celldamaging effects. High-power pulses can also create temporary cell openings, improving how well complementary therapies work together. Unlike continuous heating, pulsed heating better preserves natural body functions, promoting more effective treatment. It can also effectively reduce pain and improve patients' daily lives.

It is technically important that the pulsed heating allows precise control over treatment temperature and duration. Adjusting pulse duration, frequency, and power helps achieve therapeutic effects while minimizing harm to surrounding tissues. Constant pulse power ensures consistent energy delivery, akin to digital technology, making dosing more predictable and reducing side effects. The method's short, low-duty cycles decrease the likelihood of skin and fat tissue burns, ensuring surface temperatures remain safe. Pulsed heating's potentially higher effectiveness might allow lower overall heat doses than continuous heating, potentially shortening treatment times. Benefits vary depending on cancer type, tumor size, and location. In pulsing applications, simplified electrode applicators are possible with reduced cooling needs. The method automatically adjusts to start the semi-adiabatic heating phase without requiring stepped-up heating. Maintaining constant power throughout simplifies tuning. In practice, this method treats all depths of tissue equally.

Like all treatments, pulsed mEHT has limitations and potential drawbacks. While it reduces overall heating time, localized tissue within the treatment target can still experience high temperatures, potentially causing damage. Depending on individual factors, nerve stimulations and systemic reactions like thirst, fever, and fatigue are possible. High-power pulses, such as rapid tumor breakdown (lysis) or cytokine storm syndrome, may also pose risks.

The periodic repetition of the pulses thermally averaged by the heat-conduction of the tissues, but the nonthermal component is periodically active, there is no mechanism of averaging. This increases the safety of the mEHT treatment, avoid the thermal overloading with providing only short load of energy, and a longer relaxing period, but in the same time the nonthermal molecular activity could be increased without extra thermal load. Another factor of the safety increase is the minor thermal load on adipose tissues and skin structures, while the efficacy was considerable increase in depth of the body. The lowered thermal toxicity increases the patient's comfort during the treatment, and gives better quality of life parameters afterward.

6. CONCLUSION

The pulsed modulation mEHT increases the standard modulated electro-hyperthermia efficacy and provides safer and easier treatment. The additional highpower pulsing increases the penetration depth and, at the same time, decreases the thermal load on the fatty tissues supporting the patient's safety and quality of life. The new pulsed mEHT is a newer step forward in the increasing of safety and effectiveness of oncological hyperthermia methods. The pulsing modulation opens a new research for further optimization of mEHT using the facility of the large nonthermal electric current for electrophoretic combination. Further research may clarify the possible shortening of the treatment time due to the increased nonthermal efficacy.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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THE SYNERGY OF THERMAL AND NON-THERMAL EFFECTS IN HYPERTHERMIC ONCOLOGY

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Simple Summary: Modulated electro-hyperthermia combines the thermal and non-thermal effects of an applied electromagnetic field. This synergy allows for the selection of malignant cells with a minimal load on healthy cells. The modulated radiofrequency induces immunogenic effects, which promote the targeting of malignant cells in the system by activating the circulating tumour-specific killer cells, thereby acting as a vaccination against the tumour.

ABSTRACT

Background: Modulated electro-hyperthermia (mEHT) is unique due to its combination of thermal and non-thermal effects.

Method: This report summarizes the literature on the effects of mEHT observed in vitro and in vivo.

Results: The thermal and electrical heterogeneity of tissues allows the radiofrequency signal to selectively target malignant tissue. The applied modulation appears to activate various apoptotic pathways, predominantly leading to immunogenic cell death (ICD). ICD promotes the release of damage-associated molecular patterns, potentially producing tumour-specific antigen-presenting cells. This abscopal-type effect may target distant metastases while treating the primary tumour locally. This immune memory effect is like vaccination mechanisms.

Conclusions: The application of mEHT has the potential to expand from local to systemic disease, enabling the simultaneous treatment of micro- and macro-metastases.

Keywords: heterogenic heating; cellular selection; thermal processes; non-thermal actions; immunogenic cell death (ICD); damage-associated molecular pattern (DAMP); abscopal effect; cancer vaccination

1. INTRODUCTION

The therapeutic use of heat has a long history in medicine, epitomized by the saying, “Give me the power to produce fever, and I will cure all diseases” [1]. Historically, the aim was to achieve the highest possible temperatures for successful treatment outcomes and tumour destruction. However, without understanding the physiological and molecular effects of heat, these objectives were unrealistic. Despite advances in heating techniques, modern hyperthermia (HT) practices still rely on these early goals. Classical HT involves maintaining isothermal conditions by heating specific regions, using temperature as the sole measure of clinical efficiency.

Hyperthermia has not yet achieved widespread recognition in oncology, and even those who are actively using HT have not reached a consensus regarding its feasibility. Despite successful clinical trials [2–4] and high-level, comprehensive meta-analyses [5–8] providing convincing results, HT’s acceptance is suboptimal. This may be due to the challenges associated with the related physics [9] or physiology [10]. These obstacles can be overcome with more knowledge and research into the field [11].

To cause the desired substantial cellular damage to malignant tissue required in classical local-regional hyperthermia (LRH), temperatures above 41–42 °C are needed [12].

In practice, the varied depth of tumours and the regulation of thermal homeostasis limits the actual temperatures achieved to 39–42 °C [13]. The technical differences within LRH techniques may

result in differences in survival benefits, as can be seen when comparing separate studies on cervical carcinoma treated with radiative [14] and capacitive [15] LRH methods. A further challenge in LRH is that the non-homogeneous heating of the tumour complicates the CEM43 dose measurement (cumulative equivalent minutes at 43 °C [CEM43] [16]). To solve this dosing challenge, the area (%) that achieves the desired temperature is also considered [17]. Despite the challenges, several trials have validated oncologic HT as a curative and palliative therapy.

Electromagnetic technologies using radiofrequency (RF) and microwaves (MW) dominate the field of HT. Capacitive-coupled heating techniques use RF technology and radiative heating devices use MW technology. Historically, the goal of heating was to cause necrosis, which was shown to occur in vitro at 43 °C [18]. Both heating technologies have challenges in achieving the desired temperature safely without causing burns in the surrounding healthy tissues or the adipose layers. Magnetic Resonance (MR)-guided thermometry technology has been developed to detect and monitor hot spot formation to reduce the risk of damage to healthy tissues; however, this adds considerable cost and complexity to the treatment [19]. The selection and depth of the tumour pose additional challenges; however, these can be partly corrected by applying multiple energy sources around the body and focusing the energy absorption at the tumour location—a technique employed by rHT [20]. Targeting heat-energy-absorbing particles within the tumour provides another potential heating method that would spare the healthy tissues that do not have the heatenergy- absorbing particles. In this method, the nanoparticles are artificially inserted into the tumour [21] and targeted, heating up the malignant tissue. Methods of flushing these nanoparticles from the body after the treatment are still being investigated.

An alternate option to the nano-heating concept would be to target naturally occurring molecular groups found in higher concentrations in tumours with a particular ability to absorb more energy than other molecules [22]. The membrane lipid raft structures, which appear in higher numbers in malignant cells, have thermal stability and appear to be promising targets for heating [23]. The simulation by Papp et al. showed that the membrane rafts were excited, and the heat increased in the presence of a 13.56 MHz RF-generated electromagnetic field. In the simulation, the current density reached 23,000 A/m². The specific absorption rate (SAR) in the rafts was high, and the energy loss was therefore minimal [24].

Modulated electro-hyperthermia (mEHT) is a capacitive heating technique that employs the use of amplitude modulation of the RF wave at a frequency of 1/f. The modulation has non-thermal effects that promote the selection and apoptosis of malignant cells [25,26]. Another important difference between mEHT and classical cHT techniques, which also apply 13.56 MHz RF waves, is the precise impedance matching, which minimizes energy loss and maximizes SAR, allowing for a lower power output and improved safety. The resulting electric field has a current density low enough to excite the molecules (non-thermal excitation) without being overpowered by the resulting thermal energy production [27]. Preclinical studies on mEHT show that mEHT can trigger cell death signals, which significantly improve cellular degradation synergistically with heat. These molecular effects are triggered by the non-thermal excitation of the chemical bonds. Non-thermal excitation, which is promoted by the amplitude modulation, is not seen in classical cHT techniques [28]. The balanced synergy of the thermal and non-thermal processes appears to optimize the antitumoural effect of mEHT and has shown to promote systemic homeostatic controls such as immune surveillance for metastatic disease [29].

This review summarises the molecular and physiological heating processes achieved by mEHT, with a focus on the synergy of the thermal and non-thermal effects of electromagnetic energy absorption.

2. THERMAL LOAD

Conventional HT centres around the thermal effects and measures its success with temperature, which is a parameter of the thermal processes. The various thermal effects induce a complex set of regulatory mechanisms through homeostatic surveillance. The thermal load causes multiple reactions in the tumour, which changes various parameters associated with the tumour's interaction with the tumour microenvironment (TME). The wide range of molecular changes impacts the tumour's local conditions, and these changes cannot be described by measuring the temperature alone. Non-thermal components accompany the thermal effects [30], modifying the temperature-dependent thermal processes [31].

The thermal processes modify the chemical reaction rate (k) of a reaction with E_a activation energy. The Arrhenius law (1) empirically describes the linear dependence of $\ln(k)$ on the reciprocal of the absolute temperature ($1/T$) [32,33]:

$$k = Ae^{-\frac{E_a}{R_G T}} \implies \ln(k) = \ln(A) - \frac{E_a}{R_G} \frac{1}{T} \quad (1)$$

Where $R_G \approx 8.3 \frac{J}{mol \cdot K}$ is the universal gas constant. The cell membrane may undergo a phase transition when the lipid structure transforms from one state to another [34], which appears at a breaking point on the straight line of a logarithmic plot [35]. The reaction rate influences the parameters of the Arrhenius plot, with a substantial reduction (almost 1/3) in E_a above this point [36]. The activation energy E_a and pre-exponential factor A vary, but the linear dependence of (1) remains valid [37].

Usually, we define the thermal processes by the existence of the (1) Arrhenius plot. The break point of the plot serves as a fixed point for the HT dose. The measured break point is $T_{break} = 42.5^\circ C$ in vitro [16] and so it is used in the standardisation of HT [17], recognizing necrosis at $T_{necrotic} = 43^\circ C$.

Heat absorption and its thermal effects differ at microscopic structures that are driven by the heterogeneous electrolyte distribution, which is separated by lipid membranes. The following primary micro-absorptions modify the TME and cytosolic structures [38]:

1. Due to the different thermal and electrical properties, the temperature differs in the extracellular matrix (ECM) and cytosol. The cell membrane is not conductive when exposed to frequencies below 15 MHz due to its capacitor-like properties, which result in minimal current flow across it at these lower frequencies. This behaviour changes at higher frequencies, where the membrane becomes more conductive, allowing greater current flow and reducing the selective difference between current flow through the extracellular matrix (ECM) and the cytosol. The membrane's insulating properties dominate at frequencies below 15 MHz, maintaining distinct current pathways in the ECM and cytosol during RF heating processes. Furthermore, different current flows exist in the ECM than the cytosol,

which is isolated by the membrane during RF heating processes. When exposed to lower frequencies (<1 kHz), the cell membrane acts as a capacitor due to its lipid bilayer, which can impede the flow of the electric current. The impedance of the membrane is relatively high, which means that low-frequency fields have limited penetration and effects on the cell [39]. As the frequency increases, the impedance of the cell membrane decreases. This is because the capacitive reactance of the lipid bilayer decreases with increasing frequency. At frequencies between 1 kHz and 100 kHz, the membrane's resistance to the flow of the electric current is reduced, allowing for greater conductivity. The interaction with the fields at frequencies higher than 100 kHz becomes more complex and for frequencies in the MHz range, other phenomena, such as the skin effect, also come into play. The membrane becomes almost conductive at $f > 15$ MHz, and so at high frequencies, this type of selection difference between the current flow through the ECM and cytosol fades. The current flow heats the electrolytes differently on the two sides of the membrane during low-frequency ($f < 15$ MHz) heating. Due to its high metabolic rate, the TME conducts the current better than the ECM in the healthy host tissue; so, the temperature difference (temperature gradient) between TME and the cytosol of malignant cells is higher.

2. The heat conductivity of the membrane wall allows heat to flow from the ECM to the cytosol, forced by the temperature gradient between the two sides of the membrane [40]. However, the rapid temperature equalization throughout the membrane does not mean that the mass of the cytosol reaches the same temperature as the TME due to the larger volume and specific heat capacity of the cytosol. Studies of cellular heat responses indicate that temperature variations across cellular structures can lead to localized thermal effects, without necessarily equalizing the temperature throughout the entire cytosolic mass immediately [41]. Heating up the mass of cytosol requires a high amount of energy coming through the membrane, which takes a longer time. This is supported by the general principles of thermodynamics.

Malignant cells are highly mobile and can form temporary cell-to-cell contacts [42]. The inhomogeneous electric field appears to trigger intensive dielectrophoretic interactions in the 0.1–100 MHz intervallum [43]. The dielectric permittivity (ϵ) of the transmembrane proteins is at least two orders of magnitude higher than the membrane permittivity in which they move [44], and so the dielectrophoretic force acts selectively on them. They produce high a SAR at the contact points, causing an increase in temperature of the transmembrane protein clusters (rafts). The mechanical contacts of the cells can also absorb high amounts of energy [24].

3. NON-THERMAL LOAD

The chemical processes can also be non-thermal, involving interactions that heat cannot achieve [45]. The thermal state stabilizes the condition of the chemical machinery of living objects. The Arrhenius law (1) describes thermally induced chemical reactions. A subtle thermal effect that results in an unmeasurable temperature increase is also non-thermal when the temperature measurements are not accurate or sensitive enough. Intensive nonthermal characteristics appear where the thermal environment provides optimal conditions. The non-thermal molecular excitations point to the biomatter's natural heterogeneities [46].

The thermal effects promote chemical changes, and the modified chemical structures may cause a change in the temperature development, as appears in the Arrhenius plot's kink. Additional to the thermal changes, the electric field non-thermally modifies some chemical structures and actively manipulates the biomatter with electromagnetic interactions, like polarization, excitation, percolation, aggregation, electrophoresis, electroporation, electro-osmosis, etc. The electric field affects numerous bioprocesses, such as endocytosis [47], regulating and controlling biosynthesis [48], acting on the articular cartilage [49], playing a role in the treatment of inflammation [50], and pain management [51]. The external field may excite the transmembrane proteins, inducing signals transferred through the complex multi-component intracellular pathways. The non-thermal stimuli also impact intracellular compartments like the mitochondria [52], ER [53], Golgi apparatus, the nuclei, and the cytoskeletal network [54].

Many essential bioprocesses follow non-thermal kinetics. During a phase transition, the structure and chemical bonds of cells are rearranged [55], and although this is a thermal process, the temperature does not change during the transition. The quantum mechanics and resonances in chemical bonds are mostly non-equilibrium thermodynamic states demonstrating the synergy of thermal and non-thermal energy components [56]. The external electric field non-thermally promotes cellular fission at low [57] and high [58] frequencies, and it has promising applications in oncology [59].

3.1. Cellular Membrane

The cell membrane's electric charge (membrane potential) attracts ions from the surrounding matrix, which are essential for cell signalling [60]. These ions can form bonds with the membrane [61], altering its non-thermal functions and lowering the influence of temperature-related activities. These create a significant electrical potential difference across the membrane, which can impact how the cell operates [62].

3.2. Cancer

Non-thermal charge transfer happens at the micro and macro levels. The movement of the ions impacts the development of cancer by regulating the apoptosis–proliferation balance [63]. The collagen network in the ECM also affects the transport of the large molecules and their carried charges [64]. The non-thermal electrokinetic and electro-osmotic processes have the potential to move fluids and particles in tissues. It is therefore possible that these processes can help manage issues that are problematic for traditional heat-based treatments, such as fluid build-up from ascites, pleural effusion, and oedema [65].

Electric fields can be used to control cellular behaviour non-thermally [66]. The induced current determines the orientation [67] and the dynamics of cell division [65], and it promotes cellular migration to heal wounds [68]. Malignant cells are more negative in their charge on their surface than their healthy counterparts, and their membrane potential is markedly lower [69]. This results in a potential gradient between the tumour and its healthy surroundings [70], which produces an electric current. As occurs during the wound healing process, the generated electric current promotes and directs cell migration [71] and supports the dissemination of the malignant cells. In this way, the malignant tissue appears to mimic a wound, demanding support from the healthy host tissues [72] and behaving as if it is a chronic injury [73]. The permanent current stimulates the

proliferation of malignant cells. Feng et al. propose that this behaviour may contribute to the tumour's ability to avoid detection by immune surveillance [74].

4. THE THERMAL CONDITION FOR A NON-THERMAL LOAD

The pre-exponential factor (A) and the activation energy (E_a) in Equation (1) proportionally change in the biosystems [75,76] as follows:

$$E_a = a \cdot \ln(A) + b \quad (2)$$

where a and b are constants $a \cong 2.6 \cdot 10^3$ and $b \cong 2.5 \cdot 10^4$ [75] or $b \cong 2.6 \cdot 10^4$ [75]. The strict correlation of E_a and A shows that the thermal effects are in synergy with non-thermal components in biological media. The reaction rate is calculated from Equations (1) and (2) as follows:

$$\ln(k) = \ln(A) - \left(\frac{a \cdot \ln(A) + b}{R} \right) \frac{1}{T} \quad (3)$$

So, the slope of the Arrhenius plot becomes $s'_{Arrh} = -\left(\frac{a \cdot \ln(A) + b}{R} \right)$, instead of $s_{Arrh} = -\left(\frac{E_a}{R} \right)$.

E_a depends on $\ln(A)$, and so the dynamism of the processes (represented in A) modifies the slope. As a result, the heating speed substantially modifies the heating effects [77], the technique used influences the slope [78], and the kink position varies depending on the species in which the measurements are taken [17]. The significant differences in the biological effects of homogeneously water-bath-heated (wHT) and heterogeneously heated mEHT at the same temperature [28] indicate the complex dynamic mechanisms involved in each technique.

The empirical Arrhenius law has a chemically adequate quantum mechanical approach in which the Arrhenius law is modified [79,80], and is expressed in the Eyring formula [81]:

$$K = \kappa \frac{k_B T}{h} e^{\frac{-\Delta G^\ddagger}{RT}} = \kappa \frac{k_B T}{h} e^{\frac{\Delta S^\ddagger}{R}} e^{\frac{-\Delta H^\ddagger}{RT}} \quad (4)$$

where κ is the reaction coefficient, h is the Plank constant ($h \cong 6.63 \cdot 10^{-34} \text{ J}\cdot\text{s}$), $k_B \cong 1.38 \cdot 10^{-23} \left[\frac{\text{J}}{\text{K}} \right]$ is the Boltzman constant, and $\Delta G^\ddagger$, $\Delta S^\ddagger$ and $\Delta H^\ddagger$ are the transition Gibbs energy, transition

entropy, and transition enthalpy, respectively. In this approach, the pre-exponential factor (A') characterizes the transition state:

$$A' = \kappa \frac{k_B T}{h} e^{\frac{\Delta S^\ddagger}{R}} \quad (5)$$

The temperature remains constant during the first-order phase transition, while the pre-exponential factor changes according to the transition entropy $\Delta S^\ddagger$. The transition enthalpy (the energy in chemical systems, like bonds, solvation, etc.) depends on the final configuration but does not vary according to the process used to achieve it. The transition entropy $\Delta S^\ddagger$ refers to the unavailability of thermal energy to convert into mechanical work, which is understood as the degree of randomness or disorder in the system. These developing quantum mechanical principles [82] have impacts on a variety of applications.

The electric field can facilitate reactions or transitions by providing energy in a nonthermal manner, but with effects comparable to heating [18]. The mEHT optimizes the thermal/non-thermal synergy [83]. The external electric field non-thermally excites the transmembrane proteins using the thermal conditions to produce apoptotic signals for cell death [28]. The non-thermal component decreases the activation energy in the Arrhenius barrier (Appendix A). The selection at the cellular level is non-thermal. The electromagnetic heterogeneity of the target determines the radiofrequency signal path and thus automatically selects the highly conductive and highly dielectrically permeable microenvironment of cancer cells.

5. HEATING SELECTION OF MALIGNANT TISSUE USING INHERENT TISSUE HETEROGENEITIES

The inherent heterogeneity in malignant tissue structures, driven by differences in cancer cell size, shape, and metastatic potential, affects energy absorption. Malignant tissue can be recognized through its varying cell morphology, abnormal intercellular network, as well as dynamic properties, such as heat conduction, convection, and radiation. The variations in conductivity and dielectric permittivity can significantly change how electrical and magnetic processes occur in the tissue [84].

Due to the heterogenic complexity of malignant tissue, the same power flow [W/m²] may cause different energy absorptions depending on the given conditions [85], the organ [86], and the frequency [87].

5.1. Macro Selection

The increased metabolic activity of the malignant cells alters the TME, resulting in increased glucose metabolism (Warburg effect [88]) and a higher ionic concentration in the TME, which creates a more conductive path for the electric current. As a result, the tumour has higher conductivity (σ) than its healthy surroundings [89,90]. Furthermore, the higher water content of the malignant lesion [91] makes the ECM electrolytes more conductive [30], increasing the tumour's conductivity and enhancing the selection of the malignant tissue by the electric field.

The disorder in the malignant tissue, resulting from the broken intercellular bonds and junctions, allows for the easier polarization of molecules, increasing the tumour's electric permittivity (ϵ) and decreasing the electric impedance to further facilitate easier flow of the RF current [92]. The resulting selective RF current causes mechanical, electric, and molecular changes in the tissue [93–95].

An external field, $E_e = 10^4 \frac{V}{m} \cong 100 \frac{mV}{cell}$, creates different SAR values in the intra- and extracellular electrolytes and in the membrane. The TME $SAR_{M+TME} > 10^8 \frac{W}{m^3} \approx 10^5 \frac{W}{kg} \sim 1 \frac{pW}{cell}$ [96]. The 10 MHz range has the highest absorption rate of the cell components [96]. The selection of the tumour by mEHT is therefore possible because of the macroscopic thermal and electric heterogeneity [93]. In silico calculations reveal [24,94] that the selection of the malignant cells using the electromagnetic processes produces a 2 °C higher temperature in the deep-seated tumour target than in the surrounding tissues [97]. Chopped meat phantoms, used to imitate tumour tissue, also

showed a selective increase in temperature [98]. Experiments indicate that the cellular effects of treatment with mEHT are more enhanced than those seen with wHT at the same temperature [28,99] and in vivo [100,101]. This difference is likely driven by the synergy of the thermal and non-thermal effects. In vivo studies on murine models show that mEHT can selectively target tumours, even under challenging conditions, such as lung metastases where the heating is complicated by the air flow [101,102]. The SAR in tumours reaches 250 W/kg, while in the surrounding tissues it is 125 W/kg [94], demonstrating the macro selection by the electromagnetic field with mEHT. The macroscopic thermal effect of mEHT also makes precise tumour temperature mapping possible [94].

The energy dissipation of RF in materials is proportional to the conductivity $(\sigma \left[\frac{1}{\Omega \cdot m} \right])$ of the absorbing material and the square of the electric field strength vector (E^2). There are two calculations which can be used to determine the SAR, or the power in the absorbing volume or mass:

$$SAR \left[\frac{W}{m^3} \right] = \sigma |E|^2 \text{ or } SAR \left[\frac{W}{kg} \right] = \frac{\sigma}{\rho} |E|^2 \quad (6)$$

where ρ is the mass density of the material. Furthermore, the current density (j) is calculated as follows:

$$j = \sigma E \left(\frac{A}{m^2} \right) \quad (7)$$

Consequently,

$$SAR = \sigma |E|^2 = \frac{1}{\sigma} j^2 \left[\frac{W}{m^3} \right] \quad (8)$$

Under homogenous conditions, the measurement of j^2 offers a thermal dose in the material with σ conductivity. The thermal dose could therefore be described as follows:

$(\text{dose} = \frac{\text{energy}}{\text{mass}}) = \int SAR dt = \int \frac{j^2}{\sigma} dt, [\frac{J}{kg}]$. This simple dose monitoring equation could be applied if current matching is highly efficient and the energy loss through cooling mechanisms is minimal [18], as is the case with mEHT.

Temperature measurement is mandatory in radiative heating processes where nonselective heating is isothermal and the absorbed energy cannot be accurately calculated. The electromagnetic macro selection of tumours allows for the quantitative evaluation of the equivalent radiation dose [103], describing the synergy between radiotherapy and mEHT. This synergy has been shown in two different lung cancer cell lines, A549 and NCIH1299, and the enhancement of the equivalent radiation dose by mEHT was confirmed in mouse xenograft models [103].

It has been shown that the temperature increases with an approximately linear growth rate and without disturbing the thermal homeostatic feedback in the beginning of the treatment [104]. The SAR, in this case, is proportional to the temperature development in time (t): $\dot{SAR} \cong c \frac{dT}{dt}$.

With precise impedance matching [27], the appropriate current density (j) is achieved. In an ideal conductor, the j current density and the E field immediately follow each other, while during the polarization process, a time lag (phase difference) appears between them. The electric current j acts thermally (Joule heat), expressed in SAR, and so the absorbed thermal power density depends on j^2 (8). The non-thermal effects, such as molecular excitations, polarization, structural changes, etc., proportionally depend on the electric field E (7). E linearly drives the non-thermal processes with j , $E = \frac{1}{\sigma} j$.

Impedance-matched selective HT uses the current density j as an isodose parameter. As shown above, j does not depend on the size of the applied capacitor plates when the potential is kept constant. It is noteworthy that j depends on the reverse portion with the radius of the electrode when the power is unchanged; so, the power must be adjusted according to the electrode radius.

The applied electric field strength vector (E) creates the $j = \sigma E \left(\frac{A}{m^2} \right)$ dose according to Equation (7). Consequently, the thermal and non-thermal effects represent the nonlinear ($\sim j^2$) and linear ($\sim j$) dependences of the selective dose and the energy absorption of cancer cells. The nonlinear thermal effect ensures the general energy background, while the non-thermal effect may be resonant [105].

When j is as large as the thermal effect dominating the interactions, it renders the linear non-thermal part negligible and produces conventional homogeneous heating without selective effects. The selection of the malignant cells, without heating healthy host cells, requires a lower j , where $j > j^2$, and subsequently a relatively small ($j \leq 1$) current density. The thermal and non-thermal effects are equal when $j = 1$. This happens when the membrane rafts, the TME, and the target tissue have the same temperature and no selection, the heating becomes homogeneous, and the thermal effect dominates the complete cellular process.

The incident power determines the thermal dose by $j \sim \sqrt{P}$. Heating excites the selected molecular clusters and promotes essential immune-related processes. Maintaining the temperature compensates for the energy losses, requiring a lower dose. The unchanged temperature with a lower current density produces less apoptosis compared to the active heating periods [106]. This offers the opportunity to improve the heterogenic cell destruction using mEHT. Most of the heating processes follow a step-up heating protocol [107], gradually increasing the dose to maximize apoptosis. Apoptotic cellular degradation determines the treatment's efficacy [106]. The apoptotic processes linearly depend on the current density dose j of mEHT [108].

The absorbed thermal power (SAR) heats the membrane rafts, with the proteins absorbing more energy from the RF current than the lipid membrane [24]. The 13.56 MHz frequency directly targets the membrane lipid rafts [22], which appears as nano-heating because the absorbers, the membrane rafts, are nanoscopic.

5.2. Micro Selection

In silico models show that the induced electric field is highest in the cell membrane, which is constantly $\approx 5 \cdot 10^3$ MHz times higher than the extracellular field, and the ratio between the two

decreases by a power of $1/f$ as the waves approach higher frequencies. In the case of more realistic tissue models, the membrane's gain depends on the cell's position in the tissue, but it does not drop below 10^2 in tissue arrangements [109] and it is double that of their healthy counterparts [109]. The voltage drop at the membrane excites the raft proteins [110], triggering various processes [111]. Experiments with gold nanoparticles demonstrated mEHT's micro selection, showing higher energy absorption but decreased apoptosis due to energy sharing between membrane rafts and nanoparticles [112]. The energy-sharing phenomenon supports the theory of micro selection by mEHT.

During cytokinesis, the "neck" between daughter cells in the cell cycle's telophase → cytokinesis induces cataphoretic forces [22] and absorbs a high SAR depending on its directional position to the E field, potentially arresting cytokinesis [113]. While the temperature increases at the points of contact, there is also a significant non-thermal effect seen during cytokinesis involving the cellular connections. The tumour treating field (TTF) is a widely applied field that focuses on the non-thermal effects of fields on the cytokinetic "neck" using capacitive coupling [114]. The electric field of TTF reorients microtubules and actin fibres, inhibiting mitotic spindle assembly [115,116]. The TTF is exclusively sensitive to micro selection due to its low external power, which allows it to prevail without any dominant macroscopic (heating) effects. The electric field increases the membrane permeability [117], which promotes the uptake of chemotherapy; however, at low frequencies, the double lipid membrane completely isolates the cytosol [118].

While the TTF applies a low external power, mEHT balances the low-power thermal effects and non-thermal electric processes. Modulated electro-hyperthermia uses a higher power input than the TTF, but still lower than conventional HT [114]. The mEHT treatments have $\sim 7.5 \cdot 10^4 \text{ V/m} = 75 \text{ mV}/\mu\text{m}$ field strength effect on the rafts [24] (and the necks), which is in the range of the membrane potential, and so its impact is much higher than $\sim 2.2 \text{ mV}/\mu\text{m}$ [119].

The patient appears here as a part of a tuned electric circuit, and the target's impedance regulates the adaptation process. The low impedance of the tumour (macroscopic) and the TME (microscopic) drives the current to select the malignant cells. In this way, the mEHT achieves precise, in situ, real-time control of the intratumoural processes whilst accounting for the patient's physiology [120].

5.3. Nano Selection

5.3.1. Electromagnetic Processes

(a) Frequency Selection

Macro and micro selection target the ECM and TME, respectively. Using an RF current, the characteristic selection can be optimised [121], with the ideal range being in the MHz range [96,100]. In the context of dielectric spectroscopy and the study of biological tissues, dispersion ranges refer to different frequency ranges in which the dielectric properties of tissues change due to various relaxation mechanisms. Defining the dispersion ranges varies in the literature and this overlap is due to the wide distribution of effects that define them and their strong dependence on the target material.

Maxwell–Wagner relaxation [122] is associated with β dispersion focuses on the polarisation of cell membranes and the interfaces between different dielectric materials, such as cell membranes and the ECM [123]. This process involves the relaxation of dipoles in membrane-bound water and other components, which therefore modifies the membrane–TME interfaces, altering polarization and rearranging the charge distribution. The broad range of β -frequency dispersion effects [105] enhances the selective treatment by targeting these cellular components effectively [124]. Modulated electro-hyperthermia uses a 13.56 MHz frequency, which belongs to the medically applicable ISM band [125]. This frequency is generally associated with the second Maxwell–Wagner relaxation range, the δ -dispersion range [126], and interacts with TME-suspended particles [127], influencing organelles and protein-bound water [128].

The theoretical [96,129], and practical benefits (including antibiotic effects [130,131]) of both the β/δ dispersion ranges are well documented. Model calculations [132] show the 13.56 MHz frequency's role in these dispersion regions, leveraging the recognizable impedance characteristics [133] that impact the TME, ECM, and cancer cell membranes [134]. The β/δ dispersion ranges select malignant cells, promoting energy absorption in membrane rafts, which influence transport and signal transduction [135], are involved in numerous signalling pathways, can sense various stresses [136], and modify the response external electromagnetic fields [137]. The application in this range can therefore enhance the selection process, provided that the power output and thermal effect do not override the non-thermal effects [138]. The 13.56 MHz frequency used by mEHT promotes non-thermal interactions, targeting transmembrane proteins and the water-bound lipid–protein complexes in the rafts. Rafts, sized 25–700 nm [139], promote the interaction of the transmembrane proteins [140] and trigger intracellular signals [111], and appear to be the primary energy absorbers in the target volume, making them a potentially important focus in cancer therapies [141].

(b) Modulation

To support healthy processes, homeostatic control manages transmembrane protein excitation, promoting natural cell degradation. Large transmembrane proteins are less likely to vibrate at high frequencies, and therefore require lower α dispersion frequencies ($10 \text{ Hz} < f < 10 \text{ kHz}$) [142]. However, these lower dispersion frequencies can also stimulate muscles [143], which may be undesirable in certain contexts. To specifically target large molecules without undesirably stimulating muscle fibres and neurons, a high frequency is applied with modulation, creating an effective frequency within the α dispersion range [25]. This modulation leverages the selective advantages of β/δ dispersion frequencies for molecular excitation, enhancing the non-thermal components of absorption and promoting malignant cell degradation [105]. Experimental evidence supports the effectiveness of such modulation [25,26].

Modulation within the α dispersion range may enhance tumour-specific energy absorption [11]. Cell membranes rectify the signal [144–146], converting the alternating current to a direct current and resulting in the accumulation of signal from the external alternating current electric field. The rectified potential level is approximated to $1 \mu\text{V}$, which does not directly modify the membrane potential but could induce significant ion fluxes and cell

disequilibrium. The resistivity and capacity of the TME and cell membrane determine the time constant of the detection of stimuli and changes in the environment [11].

Autocorrelation, a statistical tool that helps assess how current signals relate to past signals in the same pathway, can be used to analyse the regularity and predictability of signals within these homeostatic pathways. Consistent patterns (i.e., positive autocorrelation) can indicate normal, healthy functioning [147,148], and the mixture of all signals in a healthy system forms a structured pattern of fluctuation, referred to as “noise” [149]. This noise is structured and maintained by homeostasis. The noise density $S(f)$, or power at a certain frequency f , decreases as the frequency increases, as described by the equation

$$S(f) = f^{-\alpha} \quad (9)$$

This relationship holds true at all levels, from molecules to the entire organism [150], and the noise density pattern is most evident at low frequencies. External signals could therefore be applied to enhance signals from these healthy pathways [151], selecting chemical reactions based on their timing and sequence to promote healthy enzymatic processes.

The α parameter in the healthy system is $\alpha \sim 1$, and in this state, the relative error of cellular fission remains low. When the system α deviates from $\alpha \sim 1$, indicating a problem in homeostatic control, the errors in cellular processes such as cellular fission increase [152]. In this way, applying Function (9) can help to differentiate between healthy and malignant cells [153,154] and the principles of chaos theory can be used to understand and control biological processes [155,156], as a healthy system will show ordered chaos [157].

Modulated electro-hyperthermia applies the signal mixture equivalent, $S(f) = f^{-1}$, as an amplitude modulation of the 13.56 MHz carrier, much in the same way frequencies are modulated to deliver radio broadcasts (audio frequency, α dispersion range) to the listeners.

5.3.2. Molecular Selection

A cylindrically shaped lipid raft with an average diameter of 100 nm and a thickness of 7 nm has a volume of $5.5 \cdot 10^{-23} \text{ m}^3$ and absorbs extremely large amounts of energy calculated in the following cubic meter raft volume: $SAR \approx 2 \cdot 10^9 \frac{\text{W}}{\text{m}^3}$.

This extreme SAR heats only a thin volume, affecting only the raft with a low power: $SAR_{raft} \approx 0.11 \frac{\text{pW}}{\text{raft}}$.

Models show that this SAR heats the raft to around $\sim 4^\circ\text{C}$ higher than its environment [24]. Comparative studies of wHT and mEHT in various cell lines demonstrate mEHT's thermal and non-thermal effects [18,22,28,31,45,55]. Rafts are highly heterogeneous and dynamic cholesterol-enriched micro-domains that participate in protein interactions and compartmentalize cellular processes [158], promoting regulatory molecule binding [159]. Rafts also trigger apoptotic signalling, such as tumour necrosis factor (TNF)- related apoptosis-inducing ligand (TRAIL) death receptors, which are integral to immune surveillance. Non-

thermal effects from mEHT excite transmembrane proteins in rafts, inducing molecular and structural changes [159,160].

Malignant cells have a denser raft population than non-malignant cells [161], enhancing their selection by mEHT. The high number of membrane rafts in malignant cells absorbs significant energy [24], leading to changes in nano-mechanical properties [162], such as increased membrane compartment motility and rigidity [163]. The external electric field stabilizes the membrane raft domains in the lipid bilayers [164,165]. This, as well as the relatively low membrane potential of cancer cells [166,167], further enhances the mechanisms for nano selection.

5.3.3. Global Gene and Protein Expression Profiles

The micro selection of mEHT also appears at the DNA level, involving DNA fragmentation [168,169]. Synergistic thermal and non-thermal effects from mEHT impact DNA repair, suppress the Ki67 proliferation marker [170], and suggest a connection between DNA double-strand breaks and mEHT treatment. Gene regulation differences between wHT and mEHT are observed in various cell lines and in vivo models [108,171,172]. The gene map shows a distinct difference in gene regulation between the homogeneous wHT and inhomogeneous mEHT treatments [108] at the same 42 °C temperature. For example, a gene analysis of human lymphoma cell lines (U937) heated to 42 °C with wHT showed stimulation of the cytoprotective gene network. The HSP105 and HSP90A genes were upregulated, inhibiting caspase activation and blocking apoptosis. In contrast, mEHT at the same temperature activated anti-tumoural activity in HSPs, with the activation of specific genes for cell death, such as EGR1, JUN, and CDKN1A. Modulated electro-hyperthermia did not activate cytoprotective pathways like wHT, instead dominantly activating the FAS, JNK, and ERK signalling pathways [108], suggesting that electromagnetic effects influenced the differences in cell fate. The special characteristics of the electromagnetic field [173] on genes in the case of mEHT force apoptosis in vivo xenografts of hepatocellular carcinoma [174], gliomas [172], colorectal [171], and isograft tumours of triple-negative breast cancer in preclinical models [170].

The upregulated genes in 4T1 triple-negative breast cancer isografts show significant alterations in stress response pathways [170]; the humoral immune response (21 genes, $p = 1.38 \cdot 10^{-5}$); serine-type endopeptidase inhibitor activity (10 genes, $p = 1.9 \cdot 10^{-5}$); complement binding (8 genes, $p = 2.41 \cdot 10^{-4}$) and extracellular matrix structural constituent binding (39 genes, $p = 4.9 \cdot 10^{-11}$) are all impressive indicators of changes. The 38 upregulated genes ($p = 1.2 \cdot 10^{-4}$) in the "response to stimulus" pathway also vary highly significantly.

A transcriptomic analysis of mEHT-treated cells revealed a genetic upregulation of E2F1 and CPSF2 and the downregulation of ADAR1 and PSAT1, leading to strong antiproliferative effects on cancer stem cells (CSCs) [172]. The increases in E2F1 and p53 levels and the decrease in the PARP-1 level caused an enhanced apoptotic signal in U87-MG and A172 cells. mEHT treatment caused a drastic reduction in the number of CD133 CSCs and suppressed malignant cell migration [172]. Lastly, the combination of the thermal effect and the ionizing radiation field inhibits the proliferation of cancer cells, involving the NR4A3 and KLF11 genes [175].

6. EFFECTS OF NON-THERMAL SELECTION

6.1. Enzymatic Effects

The external electric field interacts with transmembrane proteins and triggers ionic effects in the TME [105], influencing macro processes [176] and catalysing reactions that are frequency dependent [177]. Enzymes, which catalyse biological processes by lowering activation energy barriers, can be affected by modulation, enhancing enzymatic processes [178,179]. The modulated electric field impacts enzymatic processes [180] through electro-conformal coupling (ECC, [181,182]), which has a resonant behaviour [183]. The low-frequency amplitude-modulated carrier frequencies generate stochastic resonances (SRs), inducing various biological enzymatic reactions and polymerization processes [105], maintaining homeostatic equilibrium [184], and optimising energetic processes [185,186].

Stochastic resonance (SR) happens when a periodic signal is mixed with noise, amplifying weak signals. Saturation occurs with increasing frequency or noise intensity, necessitating optimization for maximal SR [187]. The actual resonance depends on the environmental temperature, producing conditional noise. In a healthy system, cells form a “democracy” [188], exchanging information and aiding tissue regeneration and emitting healthy 1/f pink noise [189]. Cancer cells, however, prefer autonomy, disrupting multicellularity [190–192]. In this way, the applied modulated field can affect enzymatic pathways, working against malignant processes.

6.2. Stress Proteins

Thermal stress induces protective chaperoning heat shock proteins (HSPs) [193], which prevent protein aggregation and denaturation and correct the structural damage of the proteins resulting from stress [194]. HSPs, which are expressed under various stresses, can inhibit apoptosis, promote metastasis, and influence immune responses [195], and these effects help to protect the cell. Malignant cells, which have high baseline levels of intracellular HSPs (iHSPs), use the iHSPs to adapt to stress and to survive [196].

While healthy cells develop a relatively large number of iHSPs after thermal stress (an increase in 8–10 times the normal number at 42 °C), malignant cells only increase the number iHSPs by 30–40% [197]. Healthy cells may share the absorbed energy with their neighbours through the cellular network, but malignant cells absorb it individually, making them more vulnerable to heat.

The non-thermal electromagnetic stresses also induce chaperone synthesis [198], potentially enhancing the immune response [199] and promoting the formation of intercellular bonds [200]. Modulated electro-hyperthermia increases HSP expression at mRNA [171] and protein [201] levels, as observed in murine models.

iHSP70s protect the cancer cells from apoptosis and improve their resistance to further stresses [193] but may promote cell death when dysfunctional [202]. The dual role of HSPs in malignant cells as protective or destructive agents [203,204] depends on the intra- and extracellular conditions [205].

The HSPs liberated into the ECM (eHSPs) may significantly promote anti-tumoural activity [199]. The iHSPs released into the TME have a complex translocation process, including lipid interactions

[206]. During the translocation process, an intermediate state [207] is noted, during which time the HSPs are membrane bound, forming membrane HSPs (mHSP) [208]. The mHSPs induce apoptosis [200] and, depending on the conditions, either support [209] or suppress the survival [210] of the malignant cells. mHSPs attract NK cells [211] and activate innate immune surveillance. The extracellularly released HSPs (eHSPs) deliver immunogenic information to APCs for tumour immunity. Necrotic processes also may provide eHSPs without the iHSP → mHSP → eHSP conversion.

6.3. Ion Channels in the Cell Membrane

Modulated electro-hyperthermia affects membrane potential fluctuation, which is crucial for ion transmission [212]. The electric field impacts ion transport [213], cellular signalling [214], the transmembrane potential [215,216], and DNA transfection [217,218], potentially influencing the immune system through membrane-mediated calcium signalling [219]. The synergy of thermal and non-thermal effects modifies intracellular molecular motors and ion-exchanging channels [186,212,220]. The electric field activates ion transport, such as sodium–potassium ATPase [221], and increases the intracellular calcium (iCa²⁺) concentration [11,222]. External RF exposure has an impact on erythrocyte haemolysis [223] and may activate the ion channels in the cell membrane [224,225]. The electric field may influence the immune system through membrane-mediated Ca²⁺ signalling [219] and alter the transmembrane Ca²⁺ at low-frequency electromagnetic fields [226].

A comparison of wHT and mEHT under the same thermal conditions also shows significant differences in the intercellular calcium (iCa²⁺) concentration [28,30,99,108,146]. An overload of iCa²⁺ is detrimental to the cell and may cause apoptosis. The modulated field from mEHT therefore also appears to cause an increase in the iCa²⁺ concentration, which likely contributes to the destructive effects of mEHT on malignant cells [11].

6.4. Transient Receptor Potentials (TRPs)

Temperature-sensing TRP proteins in the cell membrane detect thermal changes. Four TRP subtypes are heat-sensitive [111,227], and several subtypes regulate calcium transport through the membrane. The rafts contain TRP channels, and so when applying mEHT, the selection of the rafts may directly activate TRP regulation. Stimulation of the TRPs [99] increases Ca²⁺ influx to critically high levels [99], and the high iCa²⁺ concentration promotes apoptosis through the mitochondria-dependent intrinsic signalling pathway [228]. TRPV4 is a Ca²⁺-permeable channel that works at body temperature. The temperature modulates the activation of a diverse range of microenvironmental chemical and physical signals, functioning as molecular integrators [229]. The integrative behaviour of TRPV4 is therefore active in the presence of moderate (under 40 °C) HT processes, as it senses changes in the TME. The combination of thermal and non-thermal processes creates a complex synergy, with TRPV4 being a key target for cancer treatment. Additionally, the thermal processes have a combined effect with non-thermal electric (voltage-gated processes) and chemical (capsaicin excitation) processes.

6.5. Effect on Polymerization of the Cytoskeleton

The cytoskeleton, composed of actin and tubulin polymers, frequently deviates from its normal structure in malignant cells, affecting cell shape and motility [230–234]. The external electric field can reorganize actin filaments and potentially block cytoskeletal reorganization, stopping

cytokinesis [235] and blocking cellular fission. The modulated field may promote apoptotic signal transmission [236] by applying harmonizing noise [237]. Furthermore, voltage-sensitive phosphatases (VSPs) play a role in cytoskeletal restructuring [238], and these molecules may be influenced by the electric field to support correct network building.

Cytoskeletal fibres follow the external electric field [239] and may orient the galvanotaxis [240]. An alternating current (AC) reorganizes the cytoskeleton with an optimum resonant frequency of around 1 Hz [241]. Theoretically, stochastic resonance describes this phenomenon [105]. The cytoskeleton works like a vast and complex wiring network for signals. The pathways are continuous from start to finish, and so the polymer density must be over the percolation threshold, forming long-range connectivity in a random system [242]. A continuous giant component of the order of system size exists above the threshold, but no such connectivity exists in the system below it. The excitations and ionic species move in these giant pathways much quicker than the simple diffusion allows it [243].

6.6. Apoptotic Signals

Modulated signals can trigger apoptotic excitation of transmembrane proteins and signalling pathways [244]. The extrinsic apoptotic pathway is initiated at the TRAIL death receptor, involving FADD and FAS complexes [201]. TRAIL signalling can have both proand anti-tumour effects, depending on the TME [245]. The modulated signal directs TRAIL towards apoptotic processes through both the extrinsic and intrinsic pathways, and it activates the caspase-dependent extrinsic apoptotic pathway.

As previously described, the stress caused by the modulated field causes the overexpression of iHSP70 [246], which exhausts cell performance and facilitates apoptotic cell death [101]. Furthermore, the expressed iHSP60 proteins activate the cleaved form of Cas3 favouring caspase-dependent apoptosis [247].

Additionally, the activation of p21^{waf1} by mEHT, as observed in vitro in human Panc1 [168] and C26 mouse colorectal cell lines, in vivo in A2058 and B16F10 melanoma allografts [102,248], as well as their corresponding lung metastases [97], shows significant tumour growth inhibition. The rapid increase in the expression of the CDKN1A gene, encoding p21^{waf1}, mediates the DNA double-strand break response triggered by p53 activation [102]. This results in substantial tumour growth suppression and cell cycle arrest during metastatic melanoma growth in mouse lungs [97].

The enhancement of apoptotic signals by mEHT works synergistically with ionizing radiation, enhancing the effects of radiation therapy on cancer treatment.

6.7. Chemotherapy-Boosting Field Effects

Low-frequency external electric fields may enhance the effectiveness of chemotherapy [249] and bypass multidrug resistance [250]. These fields have been shown to increase the sensitivity of cells to chemotherapies like paclitaxel and doxorubicin [251,252]. For instance, the penetration of doxorubicin improves when combined with 50 Hz electrical stimuli in drug-resistant cancer cell lines [250].

Phase III clinical trials on newly diagnosed glioblastoma multiforme patients [253] show that tumour treating fields (TTFs) combined with temozolomide chemotherapy significantly improve outcomes with negligible side effects. In trials for recurrent gliomas, TTFs results in comparable survival rates to various chemotherapies [254].

Additionally, low-frequency modulated electric fields promote liposomal drug uptake in cell lines such as HepG2, U87MG, A459, and CT26 [255]. Liposomal targeting enhances drug delivery to malignant cells while reducing uptake in healthy tissues.

The non-thermal electroporation by an electric field creates membrane permeabilization [256–258], enhancing drug penetration [259,260]. This technique is effectively combined with chemoradiotherapy [261] and has been successfully applied in treating metastatic complications [262].

7. IMMUNOGENIC EFFECTS

While the selective heating of mEHT delivers high temperatures to malignant cells, the average temperature of the tumour remains below 40 °C. This has an immunogenic advantage: temperatures above 40 °C downregulate the cytotoxicity of innate immune attacks [263], including those by natural killer (NK) cells [264]. Beyond thermal stress, nonthermal mechanisms of electric fields, aided by applied modulation, synergistically promote a specific set of molecules known as damage-associated molecular patterns (DAMPs).

The thermal and non-thermal effects of mEHT cause an increase in reactive oxygen species (ROS) levels that peaks 3 h after the treatment, as seen in HepG2 cell lines treated mEHT. Cells treated with mEHT exhibit relative fluorescence levels (4.87) three times higher than those treated with conventional HT (1.54) [32]. This activates the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and initiates the unfolded protein response (UPR), triggering the production of the endoplasmic reticulum (ER) chaperone BiP (HSP70 equivalent) [265] to restore cellular health. In efforts to re-establish the normal structures, the UPR aims to promote apoptosis and liberate eATP as a secondclass DAMP [247].

Chaperone functions intensify throughout the cell, producing large amounts of iHSPs to protect the cell's integrity, which is often pro-tumour. However, in vitro and in vivo measurements show that mEHT induces apoptosis without comparable necrosis in malignant cells. The iHSPs have potential to transition from protective to destructive roles as malignant cells secrete HSP70 partially on the cell membrane (mHSPs) and partially into the extracellular matrix (eHSPs) [244]. In HT29 colorectal carcinoma xenograft experiments, iHSP70 levels peaked at 14 h post-mEHT treatment, returned to baseline at 48 h, and showed a second peak between 72–120 h, due to mHSP and eHSP secretion [244]. Similar observations were made in mEHT-treated B16F10 melanoma allograft experiments, with increased mHSP70 and eHSP70 levels and substantial apoptosis at 48 h post-treatment [102]. When compared to other heating methods, the levels of eHSPs at 24–48 h post-treatment were significantly higher in the cells treated with mEHT [28]. The formation of iHSP60 by mEHT [201] promotes the cleavage of Cas3 [247], inducing caspase-dependent apoptosis.

In the early 1950s, Mole RH described the abscopal effect, where the local application of ionising radiation has the potential to affect lesions outside of the treated field [266]. This effect is now gaining significant interest [267,268] and is believed to be driven by immunogenic processes.

Thermal and non-thermal energy absorption by membrane rafts triggers signalling pathways, producing ICD and DAMP molecules. The non-thermal excitation by mEHT orchestrates the temporal and spatial appearance of these molecules, potentially enabling remote actions on distant metastatic locations and disseminated tumour cells [269]. Applied modulation ensures that the correct set of DAMPs appears in the appropriate order. This has been adequately described elsewhere in the literature [116].

This spatiotemporal DAMPs induced by mEHT guide ICD in an anti-tumour direction [265]. Most of the tumour cell destruction by mEHT promotes apoptotic cell death through the immunogenic pathways, producing a set of DAMPs [101,102,108,171,196,201]. The immune system may recognize eHSP70 as an immune messenger transporting intracellular antigenic peptides to antigen-presenting cells (APCs). The genetic information carried by eHSPs drives DC maturation [270], producing active molecules (cytokines and chemokines), immune cells (NK cells and tumour-specific CD4+ and CD8+ and $\gamma\delta$ T-cells), and activating antitumour immunity [271,272].

The abscopal effect requires DCs to mature into APCs. Various pre-clinical studies have shed light on the immunogenic and abscopal effects of mEHT [97,101,273,274]. These processes determine the tumour-specific immune response, which can detect and eliminate micro- and macro-metastases throughout the body, as is seen during vaccination procedures (Figure 1).

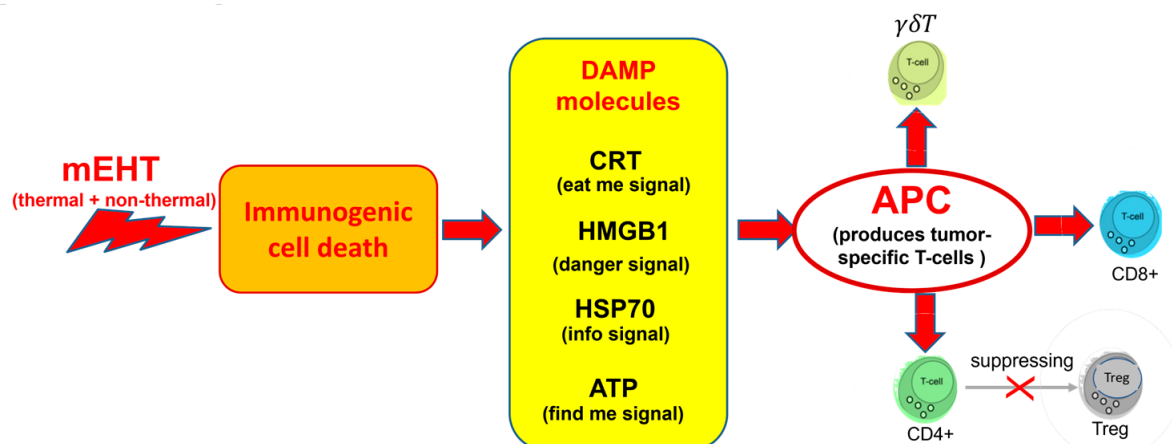


Figure 1. The combination of thermal and non-thermal effects creates immunogenic cell death, which enables the formation of a special molecular set in the TME. The appropriate molecular combination promotes the formation of antigen-presenting cells and thus enables the tumour-specific formation of killer and helper T-cells, suppressing the controversial Tregs. 8. Conclusions
Figure 1. The combination of thermal and non-thermal effects creates immunogenic cell death, which enables the formation of a special molecular set in the TME. The appropriate molecular combination promotes the formation of antigen-presenting cells and thus enables the tumour-specific formation of killer and helper T-cells, suppressing the controversial Tregs.

8. CONCLUSIONS

The complex interplay of thermal and non-thermal effects makes mEHT a unique therapeutic approach. The synergy of these effects enables the precise targeting of malignant cells, with minimal impacts on healthy tissue. The method leverages the thermal and electrical heterogeneities of tissues. The applied radiofrequency (RF) signal is modulated to induce various apoptotic pathways, creating optimal conditions for non-thermal excitation processes. This combination is fine-tuned to induce immunogenic cell death (ICD), which triggers the secretion of damage-associated molecular patterns (DAMPs). DAMPs gather immunogenic information from the tumour, facilitating the presentation of tumour-specific antigens to T-cells, enabling them to recognize and target malignant cells throughout the body. This process extends the local effects of mEHT to a systemic level, allowing the simultaneous targeting of distant micro- and macro-metastases. The locally treated tumour provides real-time immunogenic information, supporting the abscopal effect. The formation of tumour-specific memory T-cells mirrors the mechanisms of vaccination, offering a promising approach to cancer treatment.

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APPENDIX A

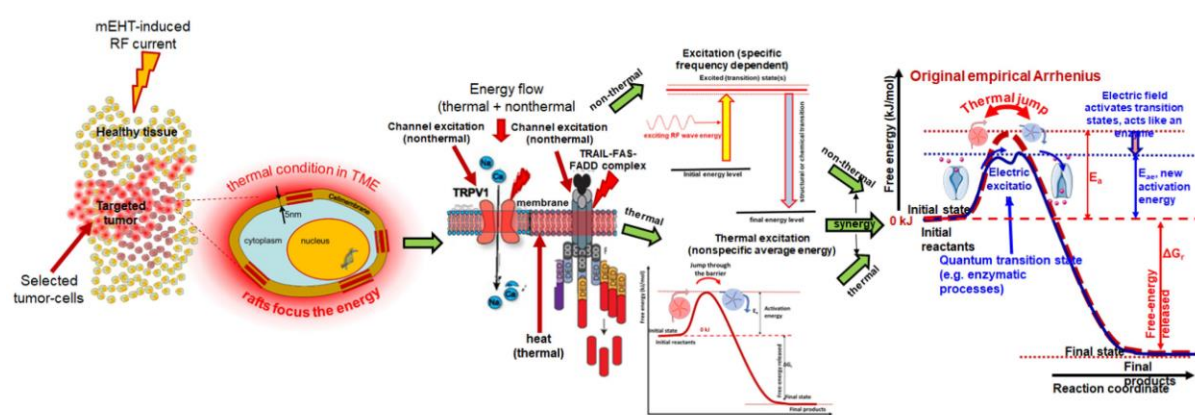


Figure A1. The thermal (conditional) and non-thermal (excitation) components selectively act on ionic transport at the membrane. The thermal energy helps to energize the ions so that they exceed the activation energy (E_a), jumping through the Arrhenius barrier. The non-thermal energy component, on the other hand, excites the appropriate signalling pathways and reduces the activation energy by promoting enzymatic processes.

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REVIEW OF PRECLINICAL DATA ON HYPERTHERMIA TREATMENT IN LYMPHOMAS AND ITS POTENTIAL FOR CLINICAL APPLICATION

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ABSTRACT

Introduction: Hyperthermia (HT) at temperatures between 39 °C and 44 °C is utilized as an adjunctive cancer therapy, serving as potent radio- and chemosensitizer. Its effectiveness in treating solid malignancies has been well established. This raises the question of whether HT can also benefit patients with nonsolid tumors, such as lymphomas.

Objective: To provide an overview of the current literature on research involving the use of HT in the treatment of lymphomas.

Material and Methods: This systematic literature review was conducted following the PRISMA guidelines. For this purpose, a MeSH-term-defined literature search on MEDLINE (Pubmed) and Embase (Ovid) was conducted from June 25 to June 28, 2024. Included were in vitro studies on lymphoma cell lines and preclinical studies on animal models with lymphoma that were both treated with HT as monotherapy or HT in combination with another treatment, and studies on patients with lymphoma. Excluded were studies that used thermal ablation and hyperthermic perfusions.

Results: Thirty-nine studies were included, predominantly in vitro studies (n = 32) or studies on animal models (n = 5). The in vitro studies utilized HT either as monotherapy (n = 6), with substances that enhance HT efficacy (n = 18) or as a sensitizer for other treatments (n = 8). Additionally, two clinical case reports on the treatment of lymphoma patients were included.

Conclusions: In vitro results suggest that HT can have anticancer effects on lymphoma cells and may enhance existing treatments. These findings are supported by in vivo studies and case reports. However, additional clinical data are needed before translation into the clinic can be implemented.

INTRODUCTION

Hyperthermia (HT) is defined as raising the temperature of tumor-loaded tissue above physiological levels, typically up to between 39 °C and 44 °C. This elevation in temperature is used as a supplementary treatment to enhance conventional cancer therapies such as chemotherapy or radiation therapy [1–3].

In vitro studies demonstrated that HT can directly and indirectly cause DNA damage and inhibit DNA repair, induce immunogenic cell death via the release of damage associated molecular patterns and increase blood flow improving oxygenation in hypoxic tumors, thereby enhancing the efficacy of chemotherapeutic drugs such as PARP-inhibitors, platinum-based agents, gemcitabine, radiation or immunomodulatory therapies [4–8].

Multiple clinical trials have shown that HT can enhance the effects of both radiotherapy and chemotherapy [9]. For instance, in patients with localized high-risk soft tissue sarcoma, combining radiofrequency HT with neoadjuvant chemotherapy resulted in increased progression-free- and overall survival compared to those who only received neoadjuvant chemotherapy. Similarly, in patients with locally advanced cervical cancer, radiofrequency HT in combination with chemoradiotherapy led to increased disease-free survival compared to the control group who only

received chemoradiotherapy [10, 11]. Van der Zee et al. demonstrated the radiosensitizing effect of HT, reporting a significantly higher overall survival ($p = 0.05$) and complete response ($p < 0.001$) in patients with locally advanced cervical carcinoma receiving HT combined with radiotherapy compared to those receiving only radiotherapy [12].

A deficit in knowledge exists regarding the use of HT in patients with non-solid tumors, such as lymphomas. Therefore, the goal of this systematic review is to assess the existing data on HT treatment in patients suffering from this disease.

METHODS

Inclusion and exclusion criteria

The systematic literature review was conducted following the PRISMA guidelines (<https://www.prisma-statement.org/>). It incorporates clinical as well as preclinical trials that used any form of HT treatment on patients with lymphoma, lymphoma animal models and human and animal lymphoma cell lines. Excluded were all studies using HT methods that induce direct necrosis, such as thermal ablation (i.e., through photothermal therapy or high-intensity focused ultrasound therapy) and hyperthermic perfusions. Only English papers published after the year 2000 were included.

Search strategy

A systematic search-term-defined literature review was conducted to find relevant resources reporting on the use HT treatment on lymphomas. The literature search was performed across PubMed (MEDLINE) and Ovid (Embase). Additionally, further literature meeting the inclusion criteria was identified through a manual search of previously gathered papers. A search in the international clinical trial registry www.clinicaltrials.gov was carried out to identify ongoing or completed clinical trials that may not yet be published. The literature search was conducted from June 25 to June 28, 2024. To ensure the inclusion of all relevant publications, the MeSH system was used for the search on PubMed, and thesaurus terms were employed for the search on Embase.

The search in MEDLINE was as follows:

(HT[MeSH Terms]) OR (HT, induced[MeSH Terms])) OR (HT, local[MeSH Terms])) OR (HT, therapeutic[MeSH Terms])) OR (heat treatment[MeSH Terms])) OR (thermal therapy[MeSH Terms])) OR (thermotherapy[MeSH Terms])) OR (thermo chemotherapy[MeSH Terms])

AND

(lymphoma[MeSH Terms]) OR (abnormality, lymphatic[MeSH Terms])) OR (lymphoid neoplasm[MeSH Terms])) OR (b cell lymphoma[MeSH Terms])) OR (lymphoma cell line[MeSH Terms])) OR (cell, u937[MeSH Terms])) OR (RAJI cell[MeSH Terms])) OR (lymphatic cancer[MeSH Terms])

Filters: From 2000–2024
and in Embase (Ovid):
HT/or thermotherapy/or thermotherapy device
AND
Lymphoma/or lymphoma cell/

As an example, a search using the thesaurus term thermotherapy includes the following synonyms: “Artificial HT, dry heat therapy, hyperthermic therapy, hyperthermic treatment, induced HT, infrared therapy, therapy, heat, thermal therapy”

Data extraction

To systematically assess the studies, they were reviewed based on inclusion and exclusion criteria, heating method, duration of heating, applied temperature, study population characteristics and whether another intervention was involved. A risk of bias assessment was not performed as it was deemed unfitting for a preclinical review.

RESULTS

Search results

The systematic search on MEDLINE (Pubmed) and Embase (Ovid) resulted in 498 publications identified on MEDLINE and 54 publications identified on Embase. After screening of title and abstract, 37 publications were sought for retrieval. Another 19 publications that met the inclusion criteria were found manually by screening the references of the identified studies. No ongoing or not yet published studies could be identified. The studies excluded after the screening of titles and abstracts were, for example, removed because they used incorrect heating methods, such as thermal ablation, or because the intervention targeted solid tumors or noncancerous tissue. 51 full-text articles were assessed for eligibility. In the end 39 articles that met the inclusion criteria were included. For an overview of the selection process see Figure 1.

Characteristics of the studies

The identified studies were categorized into three main groups: in vitro studies on various lymphoma cell lines (n = 32), preclinical studies on animal models (n = 5) and case reports on patients with lymphoma (n = 2). The in vitro studies were further divided into the following subcategories:

1. ‘Combination of potential thermosensitizer with HT’ (n = 18): – Studies that combined HT with a potential thermosensitizer to enhance the efficacy of HT.
2. ‘HT as a Monotherapy’ (n = 6): – Studies that explored HT treatment as a monotherapy.
3. ‘HT as an additive therapy to improve another treatment’ (n=9): – Studies that used HT to boost the effectiveness of a different primary intervention.

The goal of the review is to highlight key findings from the literature that are relevant for clinical translation in lymphoma treatment. However, studies that combined HT with thermosensitizers

were excluded from the main analysis due to their limited clinical relevance and are only summarized in Supplementary Table 1. The most important methodological characteristics of the included studies, as well as features of the study populations, are summarized in Supplementary Tables 2, 3, 4 and 5 based on their respective categories. One study utilized both in vitro cell lines and animal models and is therefore included in both categories.

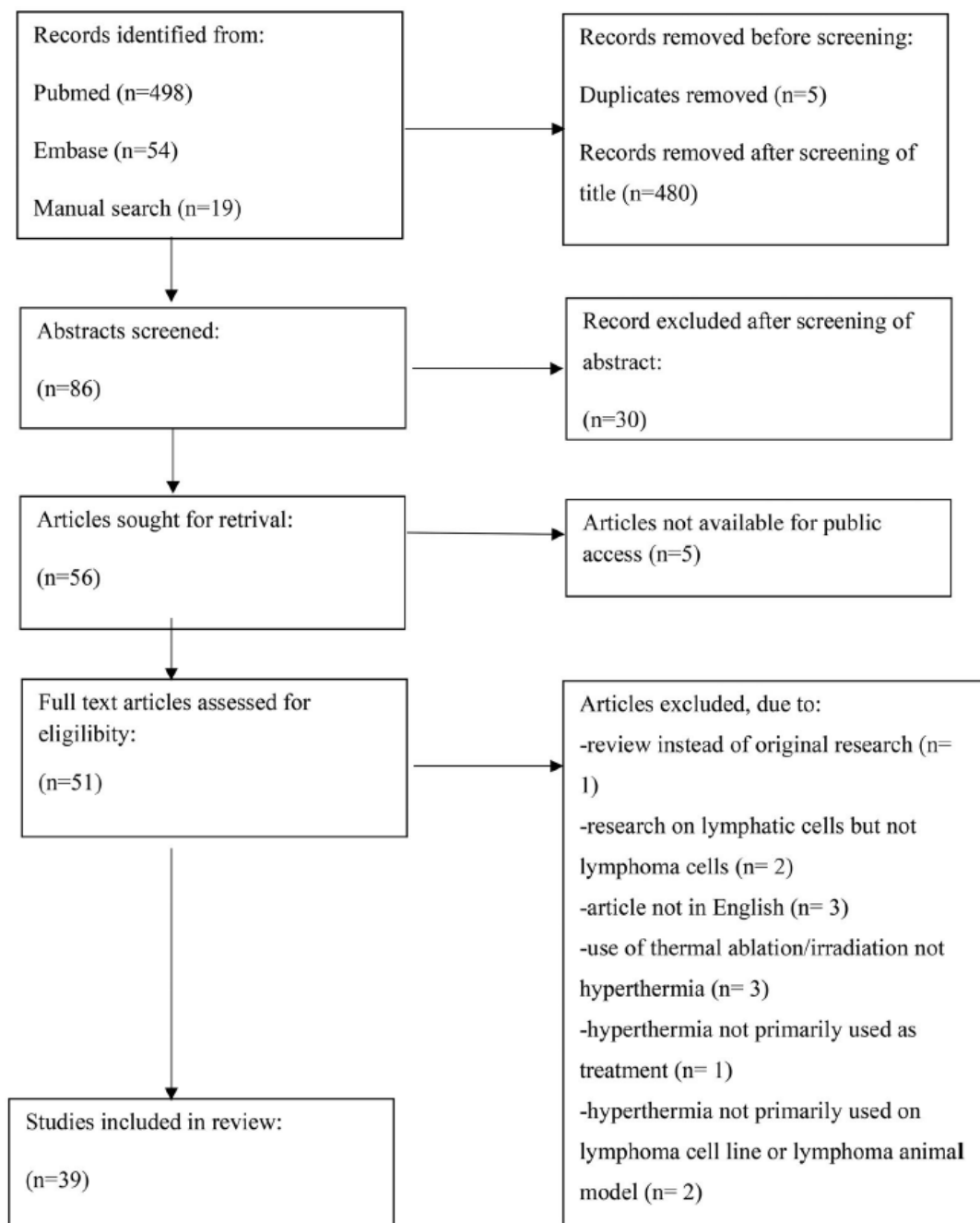


Figure 1. *Publications with titles already revealing a conflict with predetermined inclusion and exclusion criteria (i.e. use of thermal ablation methods such as high intensity focused ultrasound or thermal ablation, or the application on strictly non-cancerous tissue, the application on solid tumors, etc.) were excluded before abstract and full text screening.

Effects and molecular mechanisms of HT as a monotherapy on lymphoma cell lines

All studies (n = 6) from that group are summarized in supplementary Table 2. There are four studies [13–16] evaluating the effects of HT in the form of a water bath on U937 lymphoma cell lines (Histiocytic lymphoma cells isolated from a 37-year-old male patient) (n = 3) and chicken B-Lymphoma DT40 cells (n = 1). The effects of HT were measured by detecting apoptosis and apoptosis-related markers, as well as gene networks responsive to HT.

It was observed that HT-induced apoptosis occurs only at temperatures higher than 42.5 °C, which is described as the inflection point of HT [13]. However, the induction of apoptosis also depends on the duration of heating. In U937 lymphoma cells that were heated via water bath hyperthermia (WHT) at 42 °C, significant DNA fragmentation was only observed after heating for more than 60 min [15].

Tabuchi et al. [16] identified changes in genetic networks associated with the response to mild HT in human U937 lymphoma cells, offering new insights into cellular responses to heat stress. Specifically, it was revealed that mild hyperthermia in U937 cells resulted in increased expression of heat shock proteins (HSPs) like Hsp27, Hsp40, and Hsp70.

Furusawa et al. [13, 15], further identified HT-specific gene networks and cellular response patterns, for example, the activation of apoptosis-related gene networks in conditions of severe heat stress. They are also emphasizing on the balance between cytoprotective and pro-apoptotic genes.

A particular focus was on the expression of HSP in response to HT. HSPs are expressed in response to various cellular stressors, including heat stress. HSPs are often overexpressed in lymphomas and are associated with increased therapy resistance [17, 18]. They function as chaperones for other proteins and have cytoprotective effects by inhibiting apoptosis. However, they also have immunomodulatory effects that may enhance anti-tumor responses. HSPs have also shown to be potent immune system activators by facilitating antigen presentation, inducing cytotoxic T-cell responses, and stimulating cytokine production [19, 20]. It was shown that HSP27, HSP40 and HSP70 are significantly upregulated mediated via the heat shock transcription factor (HSF) following HT treatment at 44 °C and even after mild HT at 42 °C and 41 °C. HSPs may be key factor in inhibiting apoptosis in lymphoma cells [4–6].

Two studies compared conventional WHT to modulated electro-HT (mEHT) and presented the molecular differences in cell death mechanisms and cellular response toward HT [21, 22]. mEHT is a form of amplitude-modulated radiofrequency heating that generates heat using capacitive-coupled energy at 13.56 MHz. It was shown that the temperature-dependent cell death in WHT and mEHT were different. A significant increase in cell death was observed at 41 °C in cells treated with mEHT. After peaking at 41 °C, cell death decreased and only started to rise again at 45 °C. In contrast, significant cell death in cells treated with WHT was observed only at temperatures higher than 44 °C. The authors attribute this observation to the stronger activation of the extrinsic apoptotic pathway and reduced expression of HSPs in cells treated with mEHT compared to those treated with WHT. In summary, mEHT and WHT result in different biological responses. At lower temperatures, apoptotic-inducing pathways were dominant in cells treated with mEHT, while in cells treated with WHT cell-protective mechanisms prevailed.

These studies identified pathways and proteins that play important roles in HT-induced apoptosis and heat tolerance. This could be used as a steppingstone for further research assessing the sensitivity of lymphoma cells to HT. Moreover, understanding the mechanisms behind HT-induced apoptosis as well as the signaling pathways upregulated in response to heat is important for determining which cytostatic drugs present enhanced efficacy when combined with HT. Despite offering valuable insights into basic mechanisms of HT, these studies are not relevant for potential clinical translation. It is also uncertain to what degree these findings can be applied to other lymphoma cell lines or if they are specific to lymphoma cells.

HT as an additive treatment to cytostatic drugs and radiotherapy in lymphoma cell lines

All studies considering HT as an additive treatment to cytostatic drugs or radiotherapy in lymphoma cell lines are summarized in Supplementary Table 3.

Seven studies using different chemotherapeutic drugs in combination with HT on lymphoma cell lines were found [23–29]. Istomin et al. investigated the effect of HT on the cytostatic activity of different chemotherapeutic drugs (carboplatin, cisplatin, oxaliplatin, carmustine, etoposide and gemcitabine) on RAJI human lymphoma cells. The cells were treated with these drugs at either 37 °C or 42 °C for 15 or 30 min in combination with HT. Treatment efficacy was measured using the IC50 dose (drug concentration resulting in growth inhibition by 50% vs. the control) and the thermal enhancement ratio (TER) (IC50 dose for drug alone divided by IC50 dose for drug combined with HT). They demonstrated a synergistic enhancement in the efficacy of carboplatin (TER = 1.8–2.4), cisplatin (TER = 2.0), and oxaliplatin (TER = 3.0) when combined with HT, as well as additive effects when combined with carmustine and etoposide. HT for 15 min in combination with gemcitabine resulted in a slight increase in cell growth inhibition (TER = 1.4), whereas hyperthermia for 30 min led to a decrease in cell growth inhibition (TER = 0.8). The strongest synergistic effect was observed when platinum-based drugs were combined with gemcitabine [26]. However, Fang et al. reported that HT at 42 °C for an hour resulted in increased resistance of RAJI lymphoma cells to adriamycin and cisplatin. A possible explanation for that is the induction of HSPs by heat that have a cytoprotective effect [25]. Milani et al. and Saliev et al. described an addition of HT-induced and bortezomib-induced effects when treating U937 lymphoma cells and different mantle cell lymphoma cell lines [23, 24]. Milani et al. also demonstrated that increased levels of HSPs induced by heat do not interfere with the sensitivity of mantle cell lymphoma cells to bortezomib [23].

These findings indicate that HT has chemo and radiosensitizing [30] effects on lymphoma cells. However, the mixed results, varying study designs, which do not allow for direct comparison, and the limited number of studies, emphasize the need for further research.

In vivo studies on animal models with lymphoma

There are five studies that are using a form of HT on animal models with lymphoma [31–35], (Supplementary Table 4).

Two of them are investigating the immunogenic effect of HT on EL-4 lymphoma cells injected in C57L/6 and E.G7-OVA mice (n = 8 per group) [31, 32].

Ando et al. evaluated whether local HT can induce a tumor-specific immune response mediated through cytotoxic t-cells. C57BL/6J mice were injected with the lymphoma cell line E.G7-OVA (2.0×10^6 cells/mouse) in the right femur. The mice were then divided into four groups: a control group

that received no treatment (control), a group that received only local HT, a group treated with only immunomodulatory antibodies (anti-mouse CTLA4 mAb/anti-mouse CD8 mAb), and a group that received a combination of immunomodulatory antibodies and local HT. One week after tumor inoculation, the mice underwent local HT in the form of a water bath for 60 min at 42 °C. Treatment efficacy was evaluated by measuring tumor size and tumor growth duration (defined as the number of days taken until the tumor volume reaches 1000 mm³ following local HT treatment). T-cell activation was assessed by measuring the production of interferon- γ . The results showed that local HT significantly suppressed tumor growth duration ($p = 0.01$) and clearly induced E.G7-OVA-specific interferon- γ -producing splenocytes, suggesting a systemic antitumor response. Additionally, treatment with anti-mouse CD8 mAb significantly ($p = 0.02$) reduced the therapeutic efficacy of local HT, but had no effect on the control group, indicating that the CD8+ T-cell immune response is a component of the local HT-induced antitumor immunity. However, anti-CTLA4 mAbs did not significantly enhance the therapeutic efficacy of local HT.

Tanaka et al. utilize magnetic nanoparticles to create heat at the tumor site as a method of localized HT. When an external alternating magnetic field is applied, these nanoparticles generate heat specifically within the tumor. To investigate HT-induced immunogenic effects mouse T-lymphoid EL4 cells were injected into the right flank of C57BL/6 mice. The mice were divided into four groups, either receiving no treatment (control), HT treatment for 30 min at 45°, an injection of immature dendritic cells or HT and dendritic cells combined. In the mice receiving the combined treatment complete regression was observed in 75% compared to 12.5% in the mice that only received HT. In the mice that did not receive any treatment or only DCs no complete regression was observed. Both studies showed that HT can have an immunomodulatory effect probably through helping antigen presentation to antigen-presenting cells such as DCs leading to enhanced activation of cytotoxic T-cells (CTL). A potential mechanism for this involves apoptotic tumor cells induced by HT releasing intracellular antigens, which are then taken up by DCs. These antigens are subsequently presented on MHC class I or II molecules to T cells [31]

Dai et al. were using magnetic nanoparticles for thermochemotherapy in mice that were injected with the diffuse large B-cell lymphoma cell line OCI-LY18 [33].

The study confirmed that magnetic hyperthermia synergizes with chemotherapy by inducing intrinsic apoptosis and targeting tumor cells effectively, enhancing overall treatment efficacy while reducing chemotherapy drug usage. In five different treatment groups, the mice either received only methotrexate (MTX), only Fe₃O₄ nanoparticles, nanoparticles loaded with MTX, only nanoparticles that were heated using an electromagnetic field for 60 min or nanoparticles loaded with MTX in combination with heat treatment for 60 min. According to MRI and pathological analyses tumor volume in the Fe₃O₄ + HT group was significantly lower than in the Fe₃O₄ only group ($p < 0.05$) suggesting that HT can significantly inhibit tumor progression. The same interventions performed in vitro showed synergistic effects between chemotherapy and thermotherapy as evidenced by the upregulation of pro-apoptotic genes (Caspase-3 and Bax) and downregulation of the antiapoptotic gene Bcl-2. Masanuga et al. demonstrated a recovery inhibiting effect of HT in EL4 lymphoma cells injected into mice following radiotherapy [36].

These results suggest the efficacy of HT on animal models with lymphoma and suggest an immunomodulatory effect of HT that may be used in treatment in combination with other

immunotherapies. The low number of in vivo studies and the heterogeneous study design underscore the need for further research.

Case reports

Two case reports describing a HT treatment on patients with lymphoma were found [37,38], (Supplementary Table 5). Barni et al. describe the treatment of a 63-year-old patient with non-Hodgkin lymphoma, primarily localized in the axilla [38]. The patient underwent two sessions of capacitive RF HT with a frequency of 13.56 MHz for 90 min each, with a 72-h interval between treatments, during a relapse while receiving chemotherapy. The temperature was kept between 41–43 °C. A biopsy was taken after each treatment to assess treatment efficacy by measuring apoptosis and mitotic activity of the tumor cells, comparing these results with biopsies taken before HT treatment. The biopsy showed significant ($p < 0.01$) increase in apoptosis, and significant inhibition of cell proliferation in cells treated with HT compared to cells that were taken before treatment. Incidence of apoptotic cell death almost tripled after the second treatment. Indicating a significant reduction of the tumor growth.

Honma et al. reported almost complete regression of a primary cutaneous anaplastic large-cell lymphoma in a 89-year-old patient treated with local thermotherapy using pocket hand warmers [37]. The tumor, which was localized on the chest, recurred after resection. Local HT in the form of pocket hand warmers was applied for 2 h daily for the first seven days and then every other day. The skin temperature can approximately reach 42–43 °C using the pocket hand warmers. Treatment efficacy was assessed by measuring tumor growth and levels of blood thymidine kinase (normal range 0–5 U u/L). After 10 weeks of treatment the tumor almost completely regressed and levels of blood thymidine kinase decreased from 19 U u/L to 10 u/L. However, the results can hardly be considered significant because HT was used as monotherapy, and the patient died from pneumonia four months after the treatment began.

In both cases, the lymphoma was primarily localized, which is not representative of the majority of lymphoma patients. Furthermore, case reports lack statistical significance and, therefore, only suggest that HT might be beneficial in the treatment of lymphoma.

DISCUSSION

The results of our systematic literature review show mostly preclinical evidence suggesting anticancer effectiveness of HT in lymphomas [13]. In addition, enhancement of HT was observed using substances such as isofraxidin, cordycepin or cold atmospheric helium plasma [39–41]. However, these substances, along with the other thermosensitizers, are not relevant for potential clinical translation. Furthermore, HT synergistically enhanced the efficacy of chemotherapeutic drugs, that is, oxaliplatin, cisplatin or carboplatin as well as enhancing the effect of radiotherapy [26, 30]. In vivo studies, where diffuse large B-cell or T-cell lymphoma cells were injected in mice, supported those findings while also reporting on the immune modulatory effects of HT [31, 33].

The main modes of action of HT in lymphomas were summarized in Figure 2.

However, only two clinical case reports were identified; in addition, a search of the clinicaltrials.gov database showed no apparent ongoing clinical trial activity studying HT in hematological malignancies.

Lymphomas are malign neoplasms originating from lymphatic cells accounting for about 4% of all cancers. They are divided into Hodgkin (HL) and non-Hodgkin lymphomas (NHL). NHL can be further divided into B-cell and T-cell lymphomas, with approximately 85% being B-cell lymphomas.

First-line therapy usually consists of a combination of chemotherapy regimes, radiotherapy and targeted therapy. In relapsed or refractory disease, high-dose chemotherapy, immunotherapy or stem cell transplantation are used [42].

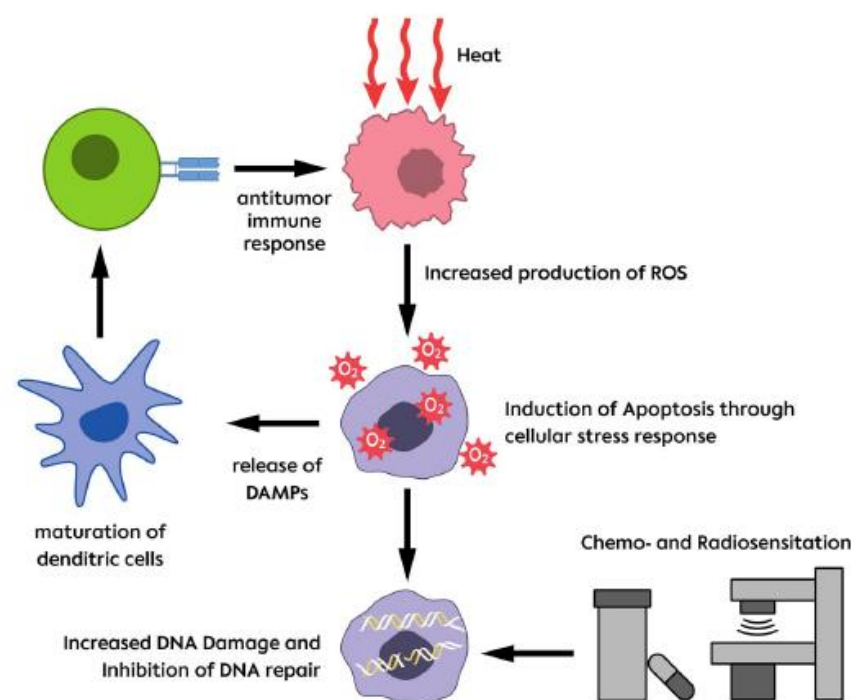


Figure 2. Heat exposure increases intracellular ROS levels, which can trigger apoptosis through a cellular stress response. Elevated ROS levels can also lead to more DNA damage and inhibit the activity of DNA repair enzymes. The DNA damage caused by HT has chemo- and radiosensitizing effects. Apoptosis induced by HT results in the release of DAMPs, which function as antigens and promote the maturation of dendritic cells. These cells, in turn, activate T-cells, enhancing the antitumor immune response. Abbreviations: ROS: Reactive oxygen species; DAMPs: Damage associated molecular patterns.

Based on the results of this literature review, several findings suggest potential benefits of HT in lymphoma treatment. Platinum-based chemotherapy, which is synergistically enhanced by HT, is currently part of the high-dose chemotherapy regime for patients with HL experiencing their first relapse, and for patients with diffuse large B-cell lymphoma (DLBCL) as part of salvage therapy combined with stem cell transplantation [26, 43, 44].

Radiotherapy, frequently used in lymphoma treatment, for example, in patients with follicular lymphoma in stages I and II or as part of first-line therapy for HL, has also been shown to be enhanced by HT in lymphoma cell lines and animal models [30, 36, 45]. The abscopal effect of radiotherapy may be enhanced when combined with HT, considering the immune modulatory effects of HT [31, 32].

Additionally, results indicate a synergistic enhancement of MTX by HT in DLBCL cells [33]. MTX is commonly used as first-line treatment in patients with primary central nervous system lymphoma [46]. Furthermore, two studies reported enhanced lymphoma cell killing when HT was combined with bortezomib, a treatment commonly used for multiple myeloma [23, 24, 47].

Lastly, considering the immune-modulatory effects of HT, there is a potential for enhancing CAR T-cell therapy as well as bispecific antibodies, a treatment option for patients with DLBCL experiencing a second relapse, when combined with HT [43].

Andocs et al. [21]: suggest that anticancer effects beyond pure temperature elevation/heating exist. This is a controversial topic. We have also found in vitro and in vivo evidence that nontemperature induced/nonthermal anticancer effects exist, in a colorectal cancer model [48]. Combined thermal and nonthermal effects of radiofrequency-based hyperthermia (for instance the mEHT approach) might be beneficial for patients. More research is required to further explore the potential of this treatment method.

The review has several notable limitations. First, it relies heavily on preclinical studies, with only two papers involving clinical trials, both of which are case reports. Consequently, there is insufficient clinical evidence to demonstrate the efficacy of HT in lymphoma patients. Furthermore, most in vitro studies explored HT either as a monotherapy or in combination with a thermosensitizer. However, these forms of application are not relevant to clinical practice. Additionally, it is unclear whether the results indicate a lymphoma-specific effect distinct from previously published findings on cancer cells.

Many studies used the U937 lymphoma cell line due to its rapid and robust response to HT-induced apoptosis, making it practical for laboratory use. However, this cell line was often employed in studies of cancer treatments more broadly, rather than being specifically targeted for lymphoma. Similarly, Masunaga et al. utilized the EL4 lymphoma cell line primarily for its strong apoptotic response. As a result, the generalizability of these findings to other lymphoma cell lines remains uncertain.

The heterogeneity among the studies further complicates evaluation. Variations in heating techniques (e.g., WHT, mEHT, nanoparticles), duration of treatment, and the cell lines used make it difficult to draw definitive conclusions. Moreover, the combination of HT with cytostatic drugs such as gemcitabine does not consistently produce synergistic effects in lymphoma cell lines, suggesting that HT may only be effective when paired with specific drugs, such as platinum-based cytostatics.

Animal studies also exhibited variability in their heating methods. A significant experimental difference was whether HT was applied to lymphoma cells before they were injected into animals or after tumors had developed. This distinction complicates the translation of results to clinical

practice, as lymphomas are generally systemic diseases, while the studies focused on localized tumors. Therefore, a key challenge lies in adapting locoregional treatments like HT for the broader, systemic nature of lymphomas.

HSPs such as hsp27, hsp70 or hsp90 can have a cytoprotective effect by inhibiting apoptosis [25]. These are commonly overexpressed in various type of lymphoma [17] and are associated with a resistance to certain chemotherapeutic drugs, for instance bortezomib [18]. A concern with HT treatment is that it may further increase the expression of heat shock proteins in lymphomas, leading to enhanced resistance to therapy.

However, the studies evaluating the role of HSPs only used HT on cell lines. Thus neglecting the immunological functions of these proteins. In other cancer cell lines, HSPs have been shown to bind antigens and facilitating their presentation to the immune system, leading to activation and maturation of dendritic cells which enhances antitumor immune responses [49, 50].

A potential approach to integrating HT into lymphoma treatment is to use its potential immunogenic effects to enhance lymphoma immunotherapy. HT can kill malignant cells in an apoptotic way that leads to release of damage-associated molecular patterns (DAMPs) such as HSPs, known as immunogenic cell death (ICD) [20]. The most important factors triggering ICD are ROS and endoplasmatic reticulum stress [6]. Additionally, DNA damage caused under HT can lead to the formation of tumor-specific neoantigens. Therefore, ICD can trigger an increased immune response via dendritic cell maturation, T-cell activation and increase of natural killer cell activity against tumor cells [51]. Through activation of cytotoxic T-cells and T-helper cells ICD can even stimulate an antitumor immune response all over the body. By amplifying an immune response HT can make the tumor microenvironment more accessible for therapies and can possibly be incorporated in lymphoma immunotherapy to enhance the effectiveness of treatments such as CAR-T-cell therapy or natural killer cell-based therapies [6, 19, 20, 51]. These immunomodulatory therapies are increasingly significant in the treatment of lymphoma. An emerging form of targeted therapy for lymphoma patients are bispecific antibodies (BsAbs) [52]. BsAbs possess two different antigen-binding sites, enabling them, for example, to simultaneously bind the CD3 antigen on CTLs and a tumor-specific antigen. This dual binding can induce MHC-independent activation of T-cells against tumor cells [53]. HT has only been utilized in combination with conventional chemotherapeutic agents on lymphoma cell lines, thereby overlooking the immunomodulatory effects of HT. Two in vivo studies on lymphoma mouse models have demonstrated that HT may have the potential to induce a CTL-mediated antitumor immune response [31, 32]. Further investigation into the combination of immunotherapy with HT would therefore be interesting to determine whether HT can provide therapeutic benefits in the treatment of lymphoma.

CONCLUSIONS

Based on the predominantly preclinical data, evidence suggests that HT treatment may have a potential anticancer effect on lymphoma. However, due to the limited number of studies and the fact that all but two concern in vitro or animal work, additional data are needed to prove the effects of HT in lymphoma. Only then can treatment schedules on how to best implement HT in current treatment strategies for lymphoma be developed. Therefore, designing clinical studies that utilize

locoregional HT in lymphoma patients is necessary to determine the efficacy of HT and identify combined HT treatment regimens that would benefit most patients.

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Data sharing

Data sharing does not apply to this article as no new data were created or analyzed in this study.

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INTEGRATIVE NATUROPATHIC TREATMENT MODEL FOR COLORECTAL CANCER: A RETROSPECTIVE STUDY

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ABSTRACT

Background: While specific elements of naturopathic medicine, such as botanical medicines and lifestyle interventions, have supporting evidence, there is limited quantitative data confirming its effectiveness as a comprehensive, whole-person medical approach for patients with metastatic colorectal cancer (CRC).

Objective: This study aims to retrospectively evaluate the integration of naturopathic modalities, including modulated electrohyperthermia (mEHT), into the standard of care for metastatic CRC. We compare survival outcomes between patients at the Integrated Health Clinic (IHC) and a matched control group from the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) database, a de-identified, publicly available cancer registry in the United States.

Methods: A retrospective chart review was conducted for 131 IHC patients diagnosed with stage IV CRC and treated with mEHT between 2010 and 2021. These patients were matched with 262 controls from the SEER database using propensity score matching. The primary outcome was overall survival, with time zero defined as the first IHC treatment date (with controls assigned the time zero of their matched IHC patient) to account for immortal time bias. Survival analysis was conducted using a Kaplan–Meier curve, log-rank test, and Cox proportional-hazards model.

Results: The overall survival analysis did not achieve a statistically significant difference (HR = .76; 95% CI: .57–1.01) between the IHC (median survival time: 29 month) and SEER groups (median survival time: 18 months). Incorporating time-varying effects, the hazard ratio (HR) for the IHC group compared to the SEER group was .63 (95% CI: .46–.86) for survival <36 months, indicating a lower hazard of early mortality in the IHC group. Moreover, IHC patients who initiated treatment within 90 days of diagnosis had significantly improved survival compared to their matched controls (HR = .45; 95% CI: .28–.70).

Conclusion: This study provides evidence that integrative naturopathic treatment, including mEHT, can significantly improve survival outcomes for CRC patients in the first 36 months post-treatment and when initiated within 90 days of diagnosis.

KEYWORDS

colorectal cancer, integrative oncology, naturopathic treatment, modulated electrohyperthermia, overall survival

INTRODUCTION

Colorectal cancer (CRC) ranks as the third most prevalent cancer and stands as the second most common cause of cancer-related deaths, accounting for approximately 9.3% of all cancer deaths globally.¹ Approximately 33% of patients with CRC will develop metastases either at diagnosis or during follow-up.² Although CRC is highly manageable when detected early, the 5-year survival rate for metastatic CRC detected in later stages remains low at 20%.³

In recent years, CRC has been on the rise among younger adults (under 50) in the United States and Canada.^{4–6} In a recent JAMA article, authors projected that by 2030, CRC will become the

leading cause of death among individuals aged 20 to 49 due to its association with aggressive tumor traits that are less responsive to treatment.⁷ These findings underscore the growing significance of CRC as a public health concern, necessitating collaborative efforts within the healthcare community to enhance patient outcomes and survival. A systemic therapy approach targeting cancer cells throughout the body serves as the mainstay treatment for metastatic CRC. Chemotherapy regimens combining fluoropyrimidines, oxaliplatin, and irinotecan have been widely used as first-line treatments for metastatic CRC, with studies demonstrating improved overall survival and progression-free survival rates.^{8,9} Immunotherapy has also shown remarkable responses in patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) tumors, leading to durable tumor control and improved survival outcomes.¹⁰

While systemic therapies can have substantial benefits, they may also be associated with side effects that can impact mental well-being and quality of life. Common side effects of chemotherapy and immunotherapies, such as fatigue, nausea, neuropathy, diarrhea, dry mouth, drowsiness and skin rashes can contribute to psychological distress and mental health issues.¹¹⁻¹⁵ Therefore, supportive care measures, including psychological interventions and integrative approaches, may be incorporated into treatment plans to address these aspects, enhance overall well-being, and potentially improve survival rates. Consequently, there exists a critical need to explore adjunctive cancer therapies that can enhance patient outcomes without undue risk.

Integrative approaches combining conventional therapies with complementary naturopathic modalities, have gained popularity among cancer patients seeking to improve quality of life and potentially enhance survival rates.¹⁶⁻¹⁸ However, evidence is lacking for its impact on overall survival, highlighting the need for further research.

Integrative oncology modalities range from dietary supplements, herbal remedies, and acupuncture to lifestyle counseling and physical therapies, aiming to support the body's natural healing processes. One of the less commonly used modalities is hyperthermia, in which body tissue is exposed to high temperatures, typically between 38.5 to 43°C, to induce a number of important physiological changes in the tumour microenvironment, and ultimately aimed at irreversibly damaging cancer cells while minimizing harm to normal tissues.¹⁹ This therapy is often used in conjunction with other cancer treatments, such as radiation therapy and chemotherapy, to enhance their effectiveness.²⁰

This approach is typically categorized into loco-regional hyperthermia, which targets specific body areas such as tumors, and whole-body hyperthermia, which raises overall body temperature to induce fever-like physiological effects aimed at addressing metastatic cancer.²⁰ Loco-regional hyperthermia delivers heat through external or internal applicators directly to the tumor site or regions of the body, whereas whole-body hyperthermia is applied systemically to effect cancer cells throughout the body.²¹ Although standard loco-regional hyperthermia shows promising effectiveness in combination with other treatments, it lacks precision at the cellular level, potentially impacting both malignant and surrounding healthy tissues.^{22,23}

To address this, modulated electro-hyperthermia (mEHT) has emerged as a unique loco-regional hyperthermia method that uses modulated and selective electromagnetic fields to generate targeted heat within cancer cells, minimizing heating of adjacent normal tissues.²⁴ This selective heating is based at least partially on the known cellular heterogeneity, aiming to exploit the

electrical properties of malignant cells and allow focused treatment of the cancerous area. The disorganized tumour microenvironment leaves sporadic cells surrounded by an extracellular matrix rich in salts and electrolytes which provide passage of the electromagnetic energy to heat the cells.²⁵ While there is some promising research on the effectiveness of mEHT in cancer populations, the evidence remains limited, primarily focused on glioblastoma, lung and pancreatic cancer.^{26–31} A search of ClinicalTrials.gov reveals only seven registered randomized controlled trials (RCTs) investigating mEHT, none of which address CRC. Additionally, studies exploring mEHT's effect on CRC has had mixed outcomes (Supplemental Table 1). While some studies suggest potential benefits, such as improved tumor regression and reduced toxicity,^{32,33} most findings show no significant improvement in overall survival compared to other therapies.^{32,34,35} The majority of studies are retrospective,^{32,33,35,36} with small sample sizes (range: 21– 62), single arm,^{33,36} and varying control treatments, limiting the reliability and generalizability of the results. The heterogeneity in study designs and lack of standardized protocols highlight a significant gap in the evidence for this treatment in this specific cancer type, CRC.

The World Health Organization (WHO) emphasizes the necessity of establishing evidence to verify the safety and effectiveness of naturopathy.³⁷ Some studies have explored naturopathic modalities like herbal medicine (eg, curcumin, resveratrol), high-dose vitamin C, and mEHT as adjunct therapies for CRC, aiming to enhance conventional treatments and improve patient outcomes.^{35,36,38–41} While specific elements of naturopathic medicine, such as herbal and nutritional supplements and lifestyle interventions, have supporting evidence, there is a limited availability of quantitative scientific data confirming its effectiveness as a whole person, comprehensive medical approach. In this study, we aim to conduct a retrospective evaluation on the integration of naturopathic modalities to the standard of care, conventional treatment. More specifically, the naturopathic treatment approach included the use of mEHT in the treatment of metastatic CRC. We assessed its effect on overall survival when compared to a matched control group.

MATERIALS AND METHODS

STUDY DESIGN

This study employs a retrospective chart review design and was conducted at the Integrated Health Clinic (IHC) in Fort Langley, BC, focused on patients treated between 2010 and 2020. The patients receiving integrative naturopathic treatment were compared to controls sampled from the National Cancer Institute Surveillance, Epidemiology, and End Results (SEER) database, a de-identified, publicly available database of cancer cases in the United States. In this study, we operate under the assumption that the standard of care, including chemotherapy, radiotherapy, and surgery, is consistent between Canada and the USA.

PARTICIPANTS

Patients were eligible for the study if they were diagnosed with CRC at 18 years of age or older and were stage IV, which meant they had metastases to other organs (eg, liver, lung, skeleton) at the time of diagnosis. The IHC patients were required to have received at least six rounds of mEHT treatments between August 1, 2010, and Dec. 31, 2021. Patients were excluded if they were missing

chart data for any of the variables used in the analysis (eg, diagnosis date, cancer type/stage, standard of care treatment, and deceased date). In general, in British Columbia, naturopathic treatment is not covered by provincial health care, so patients of the IHC must be able to finance their treatment and travel to the clinic.

Similarly, the SEER database was filtered to records with an age at diagnosis of 18 years or older and a diagnosis of stage IV colorectal cancer with complete data for all variables included in the analysis. For patients with multiple diagnoses (multiple records) in the database meeting the inclusion criteria, the record with the earliest diagnosis date was selected.

In this study, we assume that none of the SEER patients received modulated electrohyperthermia (mEHT) due to its unavailability in the United States. While several FDA-approved hyperthermia devices (<https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMA/pma.cfm>) are used in the U.S. (ie, COOK VH8500 Hyperthermia Treatment System, MEDIFOCUS 100A, PYREXAR BSD 2000, LABTHERMICS Sonotherm 1000), none of these devices provide mEHT specifically. Specific data on the use of other forms of hyperthermia for cancer is lacking; however, it is recognized that this therapy is not widely accessible in the USA.^{42,43} Regarding integrative naturopathic treatments in general, a study showed that 26.5% of insured SEER patients used complementary and alternative medicine (CAM) services, including chiropractic, naturopathic treatments, message therapy, acupuncture, and other types of CAM.⁴⁴ This variation in treatment types and their distinctiveness from the protocols offered by the IHC in this study positions the SEER data as a reasonably comparable control group, representative of the general population of cancer patients, for evaluating the specific contributions of integrative naturopathic treatment.

INTERVENTION

Patients of the IHC were treated using an integrative naturopathic treatment model, which incorporates a personalized cancer care plan tailored to each patient's needs and circumstances. This treatment model included modalities such as mEHT, intravenous therapies including high dose vitamin C, targeted supplementation, focused immune support, acupuncture, dietary and lifestyle counselling.

mEHT was applied using the EHY-2000 + Oncotherm device that can heat (via radiofrequency) tumors up to 15 cm deep to the target temperature range of 38.5–42.5°C, over 60 minutes with a treatment area of 30 cm in diameter. The EHY-2000 Oncotherm device has been approved as a Health Canada Class III Medical Device.

In addition to integrative naturopathic treatment, all patients received standard conventional care as determined by their oncologist, independent of care received at IHC. Standard care treatment may have included chemotherapy, radiation, and/or surgery. For patients receiving radiotherapy concurrently, mEHT was applied 2–3 times per week during radiotherapy, attempting to apply mEHT within the shortest time frame before or after the radiation. For those receiving chemotherapy (such as FOLFIRI, FOLFOX, FOLFIRINOX, FOLFOXIRI) mEHT was generally applied on days 1, 3 and 5 of a 14-day cycle, and on days 1, 3, 5, 8 and 10 of a 21-day cycle. In the mEHT monotherapy salvage setting, when no further surgical, radiotherapy, or chemotherapy treatments were being used or offered, mEHT treatment was typically administered 2–3 times per week. All treatments were held for a minimum of one week prior to their next scan.

DATA COLLECTION

The charts of IHC patients meeting the inclusion criteria were reviewed and their information recorded. Data were obtained through reviewing the patient's labs, pathology, imaging, consult and treatment notes from medical, surgical and radiation oncologists. The date of death was established by confirming with family members, conducting web searches for obituaries, or identifying the date when eligibility for coverage under the Medical Services Plan (MSP) was terminated. MSP refers to the public health insurance program in British Columbia, Canada, which provides provincially funded medical care for residents. Data collected and used in the analysis were: date of death, age at diagnosis, sex, year of diagnosis, stage at diagnosis, treatment with surgery (yes/ no), treatment with radiation (yes/no), and treatment with chemotherapy (yes/no). A research assistant initially pulled the data, and this information was meticulously re-checked by study principal investigator to ensure the accuracy of data retrieval. Equivalent data were extracted from the SEER database. IHC patients were followed until August 2023 to identify deaths, however, the SEER database only contains data until the end of 2021, so all IHC and SEER patients were censored at loss to follow-up or Dec. 31, 2021 if alive as of that date.

The Research Ethics Board (REB) at the Canadian College of Naturopathic Medicine reviewed and approved the study (study number CCMREBO55.Parmar). Notices were posted at the clinic locations to inform patients of the study and they had the option to opt out of participation. Participation or not had no effect on the patient's treatment.

STATISTICAL ANALYSIS

Patients in the SEER database meeting the inclusion criteria formed the pool of potential controls. Matched controls were then selected from the pool via nearest neighbour propensity score matching with a ratio of 2 controls to 1 IHC patient and a caliper of .25 in units of logarithmic standard deviation. Logistic regression was used to predict the propensity scores and the variables included in the propensity score model were age at diagnosis, sex, year of diagnosis, treatment with surgery (yes/no), treatment with radiation (yes/no), and treatment with chemotherapy (yes/no). An exact match on sex was required. Propensity score matching attempts to balance the groups with respect to measured variables to remove the influence of measured confounders. This method assumes that there are no unmeasured confounders.

For IHC patients, there was a delay between diagnosis and beginning treatment at the IHC. As such, careful handling of the baseline time was required to avoid immortal time bias. We used the prescription time–distribution matching approach to account for immortal time bias. This method sets time zero equal to the time of first clinic treatment for IHC patients and SEER patients are assigned the same time zero as their matched IHC patient. SEER patients who died before their assigned time zero were excluded. Both groups are followed from time zero until death or the end of follow-up.

Kaplan–Meier curves were used to estimate the survival probability over time per group and the log-rank test was used to test for significant between-group differences. Restricted mean and median survival times were compared between groups. A Cox proportional-hazards (Cox PH)

model was used to calculate a hazard ratio comparing the IHC patients to the controls, adjusting for remaining discrepancies between the matched groups in the measured covariates.

To investigate changes in the hazard ratio over the followup period, we re-estimated the Cox PH model with a timevarying coefficient for group. Based on the Kaplan-Meier plot and a plot of the Schoenfeld residuals, we selected a cut-off survival time of 36 months and estimated separate coefficients for group for survival times of <36 months and ≥36 months.

We hypothesized that the subgroup of IHC patients who began clinic treatment within 90 days of diagnosis would receive the greatest benefit from the intervention. To test this hypothesis, the analyses were repeated using only the subgroup of IHC patients who began clinic treatment within 90 days of their diagnosis and their matched controls. We also used a Cox PH model to directly compare survival between the group of IHC patients who began clinic treatment within 90 days of diagnosis and the group of IHC patients who did not begin clinic treatment within 90 days of diagnosis.

All analyses were performed using R Statistical Software (v4.1.2; R Core Team).⁴⁵ P-values were considered significant at a level of .05. As this study is exploratory, no multiple testing adjustment was applied.

RESULTS

There were 156 adult IHC patients diagnosed with stage IV colorectal cancer and treated with at least 6 cycles of mEHT during the study timeframe. Of these patients, 25 had missing values for at least one key variable and were removed from the analysis, leaving 131 IHC patients in the study sample. In the SEER database, 127 904 patients were diagnosed with stage IV colorectal cancer between 2000 and 2021 at age 18 or older with complete data on all required variables. From this pool of potential controls, 2 matches were selected for each IHC patient, giving a sample of 262 matched controls. After matching and the assignment of time zero, 105 controls died before their assigned time zero and were removed.

IHC patients were on average 57 years of age, with more males than females (59% male). The majority were treated with palliative chemotherapy as part of their standard of care treatment (76%) and were not treated with radiation (20%). Over half of the patients had palliative surgery as part of their standard of care treatment (60%). After matching, the SEER control group was similar to the IHC group, with standardized mean differences of less than .05 on all matching variables (Table 1).

IHC patients often had long delays between diagnosis and beginning integrative naturopathic treatment at the clinic, with a median delay of 264 days (IQR: 53–736); 63% of the sample began IHC treatment more than 90 days after diagnosis.

When measuring survival time from diagnosis, the restricted mean survival time (RMST) was 37.3 months [95% Confidence Interval (CI): 29.1, 45.5] for the SEER group and 57.2 months [95% CI: 49.6, 64.7] for the IHC group, while the median survival time was 16 months [95% CI: 13, 20] for SEER and

45 months [95% CI: 39, 57] for IHC (Supplemental Table 2). However, survival time measured from diagnosis suffers from immortal time bias due to the delay between diagnosis and the start of clinic treatment in the IHC group.

Table 1. Sample Characteristics for the SEER and IHC Groups After Matching.

Characteristic	All	SEER	IHC	P-value	SMD
Age					
Mean (SD)	56.7 (13.0)	56.7 (13.0)	56.8 (13.1)	.917	-.011
Median (IQR)	57 (49-64)	57 (49-64)	57 (49-64)		
18-24 years	2 (.5%)	1 (.4%)	1 (.8%)	.999	
25-29 years	5 (1.3%)	3 (1.1%)	2 (1.5%)		
30-34 years	8 (2.0%)	7 (2.7%)	1 (.8%)		
35-39 years	26 (6.6%)	16 (6.1%)	10 (7.6%)		
40-44 years	34 (8.7%)	23 (8.8%)	11 (8.4%)		
45-49 years	31 (7.9%)	21 (8.0%)	10 (7.6%)		
50-54 years	67 (17.0%)	45 (17.2%)	22 (16.8%)		
55-59 years	53 (13.5%)	36 (13.7%)	17 (13.0%)		
60-64 years	71 (18.1%)	47 (17.9%)	24 (18.3%)		
65-69 years	29 (7.4%)	18 (6.9%)	11 (8.4%)		
70-74 years	21 (5.3%)	14 (5.3%)	7 (5.3%)		
75-79 years	28 (7.1%)	19 (7.3%)	9 (6.9%)		
80-84 years	15 (3.8%)	10 (3.8%)	5 (3.8%)		
85-89 years	3 (.8%)	2 (.8%)	1 (.8%)		
Sex					
Male	231 (58.8%)	154 (58.8%)	77 (58.8%)	1	0
Female	162 (41.2%)	108 (41.2%)	54 (41.2%)		
Year of diagnosis					
2004	6 (1.5%)	4 (1.5%)	2 (1.5%)	1	.021
2005	3 (.8%)	2 (.8%)	1 (.8%)		
2007	3 (.8%)	2 (.8%)	1 (.8%)		
2008	22 (5.6%)	14 (5.3%)	8 (6.1%)		
2009	31 (7.9%)	20 (7.6%)	11 (8.4%)		
2010	21 (5.3%)	14 (5.3%)	7 (5.3%)		
2011	37 (9.4%)	24 (9.2%)	13 (9.9%)		
2012	46 (11.7%)	31 (11.8%)	15 (11.5%)		
2013	49 (12.5%)	33 (12.6%)	16 (12.2%)		
2014	49 (12.5%)	35 (13.4%)	14 (10.7%)		
2015	34 (8.7%)	22 (8.4%)	12 (9.2%)		
2016	19 (4.8%)	13 (5.0%)	6 (4.6%)		
2017	10 (2.5%)	6 (2.3%)	4 (3.1%)		
2018	18 (4.6%)	12 (4.6%)	6 (4.6%)		
2019	32 (8.1%)	21 (8.0%)	11 (8.4%)		
2020	13 (3.3%)	9 (3.4%)	4 (3.1%)		
Time from diagnosis to clinic treatment (days)					
Mean (SD)	—	—	538.9 (742.2)		
Median (IQR)	—	—	264 (53, 736)		
≤90 days	—	—	48 (36.6%)		
>90 days	—	—	83 (63.4%)		
Chemotherapy					
No	97 (24.7%)	66 (25.2%)	31 (23.7%)	.836	.036
Yes	296 (75.3%)	196 (74.8%)	100 (76.3%)		
Radiation					
No	320 (81.4%)	215 (82.1%)	105 (80.2%)	.748	.049
Yes	73 (18.6%)	47 (17.9%)	26 (19.8%)		
Surgery					
No	161 (41%)	108 (41.2%)	53 (40.5%)	.971	.016
Yes	232 (59%)	154 (58.8%)	78 (59.5%)		
Total (N)	393	262	131		

SMD for diagnosis year calculated as continuous.

Abbreviations: IHC, Integrated Health Clinic; IQR, interquartile range; SD, standard deviation; SEER, National Cancer Institute Surveillance, Epidemiology, and

End Results; SMD, standardized mean difference.

Using the prescription time–distribution matching approach and measuring time in months from the beginning of clinic treatment, there were no significant differences between the SEER and IHC groups in RMST (SEER: 36.7 [95% CI: 30.0, 43.4]; IHC: 37.9 [95% CI: 31.6, 44.3]) or median survival

time (SEER: 18 [95% CI: 15, 24]; IHC: 29 [95% CI: 19, 39]), as shown in Supplemental Table 2. The Kaplan–Meier curve in Figure 1 also shows no significant differences between the two groups (log–rank test P–value = .41). The Cox PH model gave a hazard ratio of .76 (.57, 1.01) comparing the IHC group to the SEER group (Table 2). This indicates a 24% lower hazard of mortality among the IHC patients compared to the SEER patients, but this difference is not statistically significant.

When the Cox PH model was re-estimated with a time varying coefficient for group, the hazard ratio comparing the IHC to SEER group was .63 (95% CI: .46, .86) for survival time <36 months and 2.02 (95% CI: 1.01, 4.03) for survival time ≥36 months, indicating a lower hazard of death among IHC patients within the first 36 months of starting clinic treatment (Table 3). This result is visible in the Kaplan–Meier plot (Figure 1), where the curve for the IHC group is initially higher than that of the SEER group, before the two curves converge.

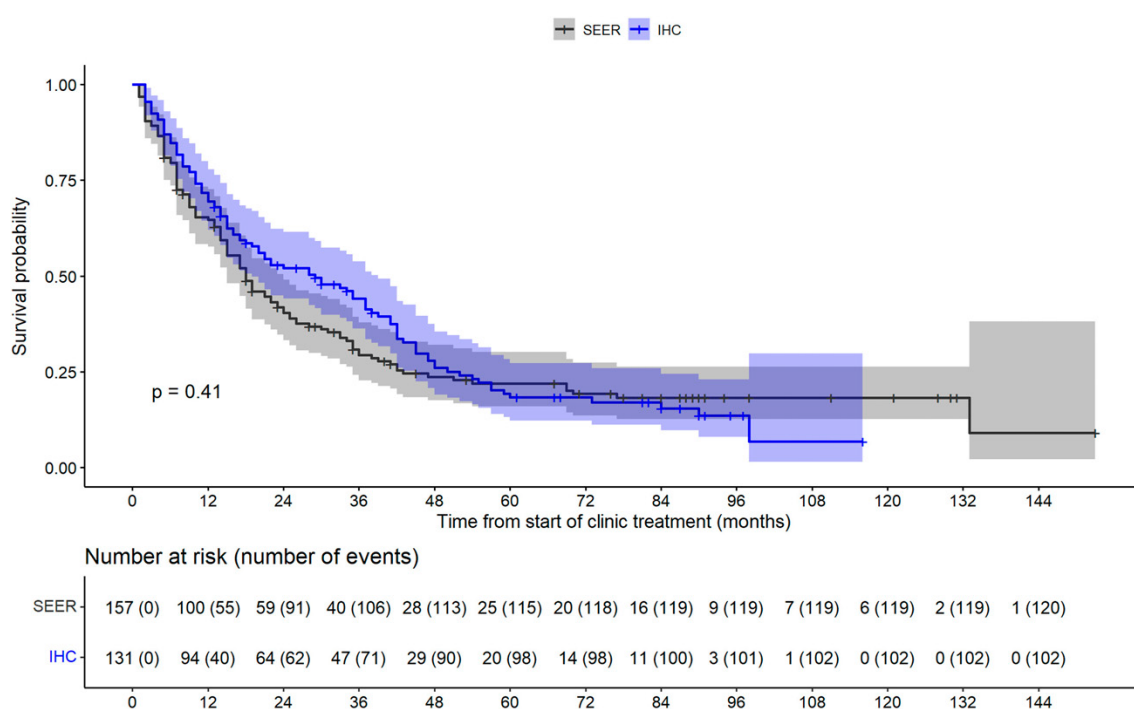


Figure 1. Kaplan–Meier curve with time measured from the start of clinic treatment for IHC patients and from the start of clinic treatment of their matched IHC patient for SEER controls.

In a subgroup analysis comparing IHC patients beginning clinic treatment within 90 days and their matched controls, the Kaplan–Meier curve shows that the IHC patients have significantly higher survival probabilities (log–rank test P–value = .015; Figure 2). When a Cox PH model is applied to this sample, the hazard ratio is .45 (.28, .70), indicating 55% lower hazard of mortality among IHC patients beginning treatment within 90 days compared to their matched controls, which is statistically significant (Table 2).

As the subgroup analysis of 131 patients revealed a survival benefit compared to controls when treatment began soon after diagnosis, we conducted a comparison of survival for IHC patients beginning clinic treatment within 90 days of diagnosis to IHC patients who began clinic treatment after 90 days. The hazard among patients beginning treatment after 90 days was 1.79 (95% CI:

[1.09, 2.95]) times greater than among patients beginning treatment within 90 days (Supplemental Table 3).

DISCUSSION

This retrospective study presents evidence that integrative naturopathic treatment can significantly improve survival outcomes for patients with CRC in the first 36 months posttreatment and when it started within 90 days of diagnosis. The median survival time from beginning clinic treatment of 29-months for patients treated at the IHC notably surpasses the 18-month median survival time in the SEER control group. This 11-month increase in median survival, although not statistically significant, is clinically relevant, suggesting that the integrative approach could be a valuable addition to the standard CRC treatment protocol. By introducing a timevarying coefficient into the Cox PH model, we observed a statistically significant lower hazard of mortality for IHC patients within the first 36 months of diagnosis; the hazard after 36 months increased to be greater in the IHC group than the SEER group. This suggests that some early deaths in the IHC group were delayed compared to the SEER group. In addition, the subgroup analysis revealed that patients who commenced the integrative naturopathic treatment model within 90 days of diagnosis had a 55% reduction in the hazard of death compared to the SEER group. This further underscores the potential benefits of early intervention.

Table 2. Hazard Ratios From a Cox Proportional-Hazards Model, for the Full Sample and Subgroup Beginning Clinic Treatment Within 90 days of Diagnosis With matched Controls. Survival Time is Measured From the Start of Clinic Treatment for the IHC Group and From the Start of Clinic Treatment of Their matched IHC Patient for the SEER Controls.

	HR (95% CI)	HR (95% CI)
	Full Sample	Subgroup: IHC Treatment ≤90 Days
Group		
SEER	Ref	Ref
IHC	.76 (.57, 1.01)	.45 (.28, .70)
Sex		
Male	Ref	Ref
Female	.79 (.59, 1.06)	.79 (.51, 1.21)
Chemotherapy		
No	Ref	Ref
Yes	.68 (.48, .97)	.53 (.33, .85)
Radiation		
No	Ref	Ref
Yes	.97 (.68, 1.39)	1.52 (.85, 2.72)
Surgery		
No	Ref	Ref
Yes	1.32 (.97, 1.79)	1.29 (.84, 1.96)
Age	1.01 (1.00, 1.03)	1.02 (1.00, 1.03)
Diagnosis year	.99 (.95, 1.04)	1.02 (.95, 1.09)

Full sample: N = 288, number of events = 222; Subgroup: N = 138, number of events = 102.
Abbreviations: CI, confidence intervals; HR, Hazard Ratio; IHC, Integrated Health Clinic; Ref, reference level; SEER, National Cancer Institute Surveillance, Epidemiology, and End Results.

Table 3. Time-Varying Hazard Ratios for Survival: Comparison of IHC and SEER Groups Using the Cox Proportional Hazards Model.

	HR (95% CI)
Group (survival time <36 months)	
SEER	Ref
IHC	-.63 (.46, .86)
Group (survival time ≥36 months)	
SEER	Ref
IHC	2.02 (1.01, 4.03)
Sex	
Male	Ref
Female	.79 (.59, 1.06)
Chemotherapy	
No	Ref
Yes	.70 (.49, 1.00)
Radiation	
No	Ref
Yes	.98 (.69, 1.41)
Surgery	
No	Ref
Yes	1.21 (.89, 1.65)
Age	1.01 (1.00, 1.02)
Diagnosis year	.99 (.94, 1.03)

N = 288, number of events = 222.

Abbreviations: CI, confidence intervals; HR, Hazard Ratio; IHC, Integrated Health Clinic; Ref, reference level; SEER, National Cancer Institute Surveillance, Epidemiology, and End Results.

We believe that the personalized nature of the integrative naturopathic treatment approach played a significant role in CRC patient outcomes. In our study, each patient's treatment plan was tailored to their specific clinical context, incorporating a combination of mEHT with other integrative modalities, such as dietary adjustments, lifestyle changes, and mind-body practices. Among the therapies utilized, mEHT was the only consistent intervention across all patients. While this personalized treatment plan limits our ability to study the effects of a single treatment modality, it highlights the adaptability of the integrative model, which seeks to address the complex and unique aspects of each patient's condition.

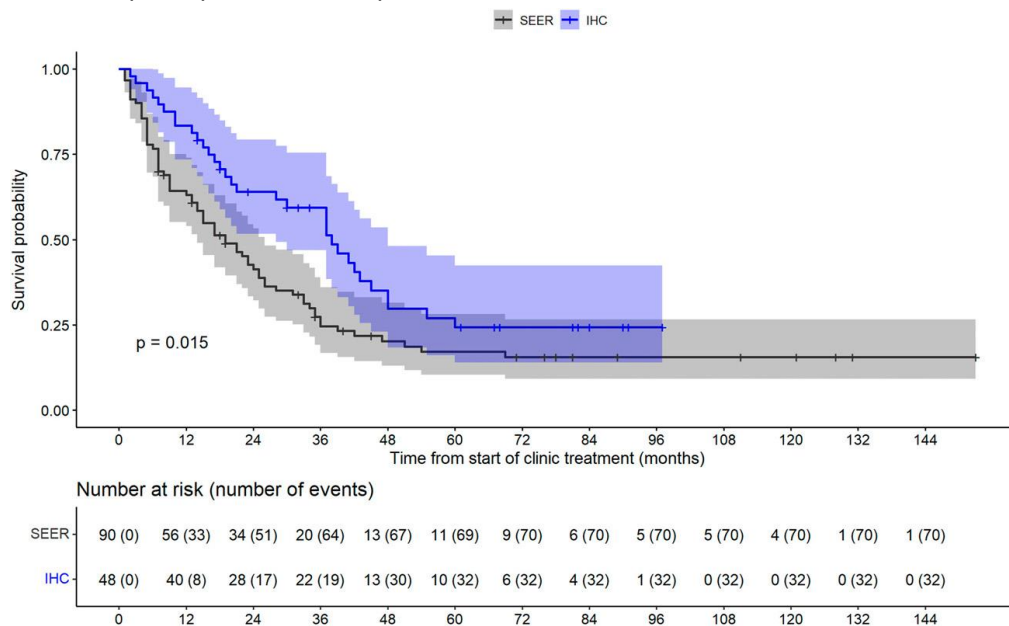


Figure 2. Kaplan-Meier curve with time measured from the start of clinic treatment for IHC patients and from the start of clinic treatment of their matched IHC patient for SEER controls, for

the subset of IHC patients beginning clinic treatment within 90 days of diagnosis and their matched controls.

The results of our study align with the previous research indicating no significant change in overall survival following mEHT.^{32,34,35} Although no survival benefit was observed, one study highlighted other potential advantages of mEHT, including improvements in tumor regression and reduced toxicity, suggesting that mEHT may have therapeutic effects beyond overall survival.³² Similarly, in the current study, we identified a time-dependent survival benefit. Specifically, patients who initiated integrative naturopathic treatment within 90 days of diagnosis exhibited a significant survival advantage compared to their matched controls, with a 55% reduction in the hazard of mortality. In a similar study, no time-dependent survival benefit was observed.³⁵ The limited sample size (n = 21) for CRC in that study may have reduced the ability to detect a significant effect in this subgroup analysis.³⁵

While research on the effect of mEHT on CRC is limited, it has been more widely studied in glioblastoma, lung, and pancreatic cancers.²⁶⁻³¹ A recent meta-analysis found that adding mEHT to standard chemoradiotherapy significantly improved survival rates in glioblastoma patients, with 1-year survival rates of 42–61% for mEHT-treated patients.²⁶ Similar to our study, this meta-analysis reported time-dependent survival benefits, with the greatest benefits observed in newly diagnosed cases.²⁶ While this study focuses on glioblastoma, it suggests that mEHT may enhance treatment outcomes in other cancers, including CRC.

A few preclinical studies have demonstrated mEHT anticancer mechanisms in CRC through multiple pathways. mEHT has been shown to upregulate heat shock proteins and enhance damage-associated molecular pattern (DAMP) signaling, which promotes immune recognition of tumor cells.⁴⁶ Additionally, mEHT induces p53-mediated apoptosis and cell cycle arrest, which further supports the efficacy of chemotherapeutic agents in CRC cells.⁴⁷ Moreover, in vivo, mEHT contributes to both localized and systemic tumor destruction in CRC models, suggesting its potential for robust anti-tumor effects beyond the immediate treatment site.⁴⁸

Moreover, our results support the use of integrative oncology including mEHT for CRC patients, but it does not provide evidence on the difference between mEHT and other forms of hyperthermia. Previous research indicates that other forms of hyperthermia, when combined with radiotherapy and chemotherapy, can significantly enhance radiosensitivity and reduce tumor size in patients with metastatic CRC.^{49,50} Future studies can compare the difference between mEHT and other forms of loco-regional hyperthermia to improve health outcomes of this population.

In addition to mEHT, other integrative approaches have been investigated for their potential benefits in cancer treatment. Recent systematic reviews of complementary and integrative therapies, including acupuncture, yoga, herbal medicine, and mind-body interventions, highlighted their role in alleviating cancer-related symptoms and improving patients' overall well-being.^{18,51-53} These findings are consistent with our study, which suggests that incorporating integrative naturopathic treatments can lead to enhanced patient outcomes.

The significant survival benefit identified by the subgroup analysis is supported by previous research that emphasizes the importance of early intervention in cancer treatment and metastatic CRC prognosis.⁵⁴ Although not specific to integrative naturopathic treatment, a delay of even four

weeks in cancer treatment is associated with increased mortality for surgical, systemic, and radiotherapy treatments across seven types of cancer, including CRC.⁵⁴ This suggests the necessity of investigating the effect of treatment delays on survival outcomes for integrative naturopathic treatments. Moreover, patient-reported outcomes, such as quality of life measures, should be utilized in future studies to determine if the quality of life for integrative naturopathic treatment patients improves beyond their survival outcomes.

The discrepancy between the insignificant result in the whole group analysis and the significant improvement in survival in the subgroup analysis results may be explained by several factors. The heterogeneity within the whole group may dilute the effects of the integrative naturopathic treatment. Patients with different treatment histories, metastatic locations or timelines and diverse co-occurring health conditions may obscure the treatment's efficacy. Alternatively, the discrepancy between the whole group and subgroup results may be due to the treatment only having an effect when applied early. Further research with larger samples is required to identify the effect of integrative naturopathic treatment on CRC patients seeking treatment at all times after diagnosis.

One of the strengths of this retrospective study is the use of a large control group from the SEER database, which enhances the validity of our findings. Moreover, to ensure robust data analysis in our study, we implemented advanced statistical methodologies. We used a technique called propensity score matching to select control patients from a large cancer database, ensuring they were similar to our treatment group on important potential confounders like age, sex, and treatment history. This method helps balance the groups and reduce the effect of measured confounders. We also carefully handled the survival time to avoid immortal time bias. We then compared survival times using established statistical methods to determine if our treatment had a significant impact. Our approach aims to provide reliable and unbiased results, demonstrating the effectiveness of our integrative naturopathic treatment model.

This study supports the effectiveness of an integrative naturopathic treatment model when implemented early following diagnosis. The use of a well-documented control group and advanced statistical methodologies strengthens the validity of these findings, aligning with the principles of evidence-based medicine. Integrative naturopathic treatment model's emphasis on combining conventional and complementary treatments can enhance therapeutic efficacy and patient well-being when implemented soon after diagnosis, paving the way for more holistic, evidence-based and patient-centered cancer care.

In the context of the Canadian healthcare system, integrative naturopathic treatment presents unique challenges and opportunities. While Canada's universal healthcare system provides comprehensive coverage for conventional cancer treatments, complementary and integrative therapies, including naturopathic treatments, are often not covered. This can create barriers for patients seeking integrative care, as they must bear the financial burden themselves. However, the potential benefits observed in our study highlight the need for policy changes to support the integration of these therapies into the mainstream healthcare system and health insurance programs.

Future research should focus on prospective, randomized controlled trials to further validate the effectiveness of integrative naturopathic treatment in metastatic CRC, and the necessity of early treatment. Investigating the biological mechanisms underlying the observed survival benefits and

evaluating patient-reported outcomes (eg, health-related quality of life) will also be crucial in understanding the full impact of these therapies on patients' overall wellbeing.

Conducting a cost-effectiveness analysis to determine if public funding by governments leads to cost savings represents a subsequent step in the research on naturopathic treatments. Expanding coverage to include integrative naturopathic treatments could enhance the overall quality of cancer care in Canada. By providing patients with access to a wider range of therapeutic options, the healthcare system can better address the physical, emotional, and psychological needs of cancer patients. The inclusion of mEHT and other integrative treatments could potentially reduce healthcare costs in the long term by improving patient outcomes and reducing the need for more intensive treatments. Therefore, advocating for policy changes and increased research funding is essential to facilitate the adoption of integrative oncology within Canada's healthcare framework.

LIMITATIONS

There are several limitations to consider. The retrospective design and reliance on chart reviews may introduce selection and information biases. We attempted to address these issues by double-checking extracted data and conducting a robust group matching strategy. The personalized treatment plan and variation in the use of integrative treatments between patients prohibits the study of the effects of specific interventions on survival outcomes. Additionally, we were unable to account for the self-selection bias in our IHC cohort due to the requirement that IHC patients must finance their treatment and travel to attend treatment at the clinic; comparable data on socioeconomic status was not available for both groups, therefore, it could not be accounted for in the analysis. The financial ability to access naturopathic treatments may limit the generalizability of our findings to broader populations. While we can be confident that very few SEER controls received any form of hyperthermia, as no devices that provide mEHT are approved for use in the USA and the use of other hyperthermia devices was found to be limited,^{42,43} it is possible that some of the SEER controls received other naturopathic treatments.⁴⁴ If that is the case and the naturopathic treatment did not have a negative effect on the patient's survival, then our estimates would be conservative estimates of the true effect obtained from comparing a group that received integrative naturopathic treatment to a group that did not receive any naturopathic treatment. Addressing these limitations with a prospective randomized clinical trial would provide more definitive evidence.

CONCLUSIONS

The observed survival benefit in the IHC group in the first 36 months post-treatment and among those who began treatment within 90 days of diagnosis, underscores the potential of integrating naturopathic treatments to complement conventional cancer therapies. This approach views the person as a whole, addressing not only the physical but also the emotional, psychological, and social aspects of a patient's life. Incorporating mEHT and other integrative modalities into standard oncological care could provide a holistic approach to managing metastatic CRC, potentially improving patient outcomes and quality of life. The integration of these treatments into clinical practice requires a collaborative and timely effort among healthcare providers to ensure that patients receive comprehensive and personalized care.

In conclusion, our study contributes to the growing body of evidence supporting the use of integrative naturopathic treatment, including mEHT, in the management of metastatic CRC. If these findings are confirmed through larger, prospective studies, they could lead to significant changes in the management of CRC, offering more hope to patients who are battling this challenging disease.

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Author Contributions

Conceptualization, G.P., M.E. and S.D.; methodology, G.P., S.I.N., and L.M.; formal analysis, L.M.; writing—original draft preparation, S.I.N.; writing—review and editing, G.P., S.I.N., S.D., L.M.; supervision, G.P.; project administration, M.E., S.D.; funding acquisition, S.I.N., M.E., G.P. All authors have read and agreed to the published version of the manuscript.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors wish to disclose that one of the authors, Dr. Gurdev Parmar, is a Co-founder and Medical Director of the Integrated Health Clinic from which the data for this study were extracted and analyzed. Although every effort has been made to ensure the integrity and objectivity of the research, the potential for conflicts of interest exists. The findings and conclusions presented in this study reflect the views of the Integrated Health Clinic and its affiliated staff. The donors/funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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ETHICAL STATEMENT

Ethical Approval The Research Ethics Board (REB) at the Canadian College of Naturopathic Medicine reviewed and approved the study (study number CCNMREBO55.Parmar).

Consent to Participate

Patient consent was waived due to nature of the study, which involved the secondary use of data.

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THE COMPLEXITY OF MALIGNANT GLIOMA TREATMENT

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SIMPLE SUMMARY

This review describes the dynamic influence of the tumor microenvironment during treatment of malignant glioma. The mechanism behind five hallmarks are outlined: glioma stem-like cells in particular (GSCs), vascularization and hypoxia, metabolic reprogramming, tumor-promoting inflammation and sustained proliferative signaling. A multimodal immunotherapy treatment plan is proposed, explaining how each hallmark can be targeted over time. Repeated tumor monitoring is deemed vital to alter the treatment plan when needed.

ABSTRACT

Malignant glioma is a highly aggressive, therapeutically non-responsive, and deadly disease with a unique tumor microenvironment (TME). Of the 14 currently recognized and described cancer hallmarks, five are especially implicated in malignant glioma and targetable with repurposed drugs: cancer stem-like cells, in general, and glioma stemlike cells in particular (GSCs), vascularization and hypoxia, metabolic reprogramming, tumor-promoting inflammation and sustained proliferative signaling. Each hallmark drives malignant glioma development, both individually and through interactions with other hallmarks, in which the TME plays a critical role. To combat the aggressive malignant glioma spatio-temporal heterogeneity driven by TME interactions, and to overcome its therapeutic challenges, a combined treatment strategy including anticancer therapies, repurposed drugs and multimodal immunotherapy should be the aim for future treatment approaches.

KEYWORDS: tumor microenvironment; malignant glioma; immunotherapy

1. THE IMPACTFUL TUMOR MICROENVIRONMENT

The significant advancements in cancer biology is reflected in the discovery and description of the 14 hallmarks of cancer [1–3] (Figure 1). In 2000, the first 6 hallmarks were summarized [1]:

- (i) acquired capabilities for sustaining proliferative signaling,
- (ii) evading growth suppressors,
- (iii) resisting cell death,
- (iv) enabling replicative immortality,
- (v) inducing or accessing vasculature, and
- (vi) activating invasion and metastasis. In 2011 [2], four novel hallmarks were defined:
- (vii) deregulating cellular metabolism,
- (viii) avoiding immune destruction,
- (ix) tumor-promoting inflammation, and
- (x) genome instability and mutation.

These latter hallmarks marked the recognition that a tumor process is not only characterized by intrinsic tumor cell characteristics but also by its specific tumor microenvironment (TME). In 2022 [3], four additional hallmarks were included:

- (xi) nonmutational epigenetic reprogramming,
- (xii) polymorphic microbiomes,
- (xiii) senescent cells, and
- (xiv) unlocking phenotypic plasticity (Figure 1).

Again, these added hallmarks refer to the influence of the TME on tumor cell biology.

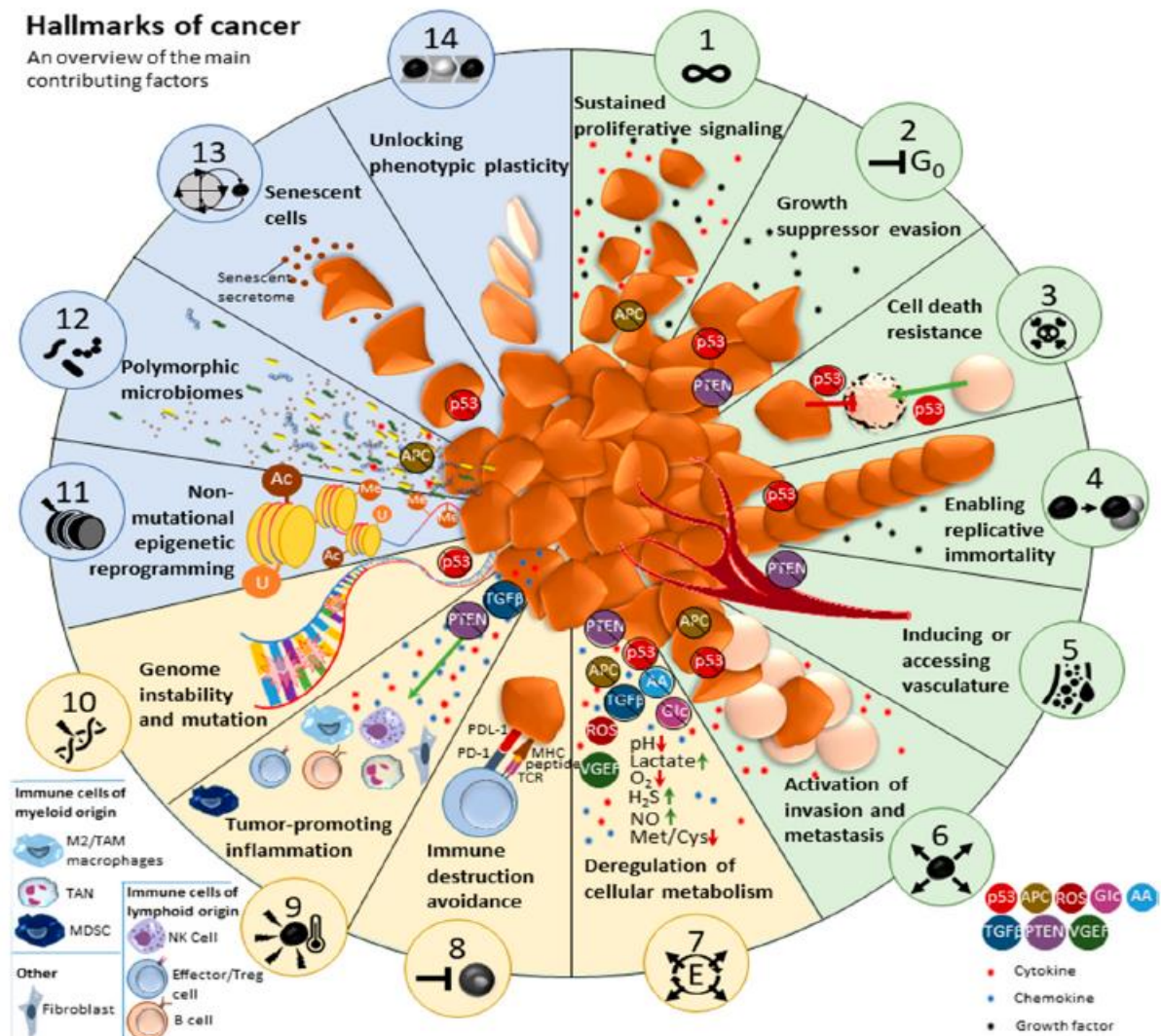


Figure 1. Top: An overview of the main contributing factors to the 14 hallmarks of cancer [1–3]. In green, the first hallmarks described. In yellow, hallmarks recognized and added in 2011. In blue, four additional hallmarks added in 2022. Legends on the lower left and right side indicate cell types, cytokines, chemokines, growth factors, and other factors vital in tumor development. Dark brown cells of irregular shapes represent tumor cells. Each hallmark has been given an icon, indicated by the numbered images in circles at the end of each hallmark pie slice. These icons will be used to indicate each hallmark in further figures.

Clearly, the role of the TME should not be underestimated. As the source of immune cells, nutrients, oxygen, extracellular communicative signals, polymorphic microbes, and as the boundary between the tumor and the rest of the body, the TME must be prioritized in ongoing and future research. Particularly, the difference in TME between individual patients, its temporal plasticity, and how it is affected by different treatment options, should be considered in future anticancer treatments.

The categorization of tumors of the central nervous system (CNS) is changing, as it has over decades, based on gained insights in tumor biology and pathology [4]. Glioblastoma (GBM), IDH-wildtype, belongs to the category of adult-type diffuse gliomas and is a highly aggressive and infiltrative, mutative, therapeutically non-responsive, complex located, and deadly disease, which

make it difficult to treat. GBM is the most frequent primary brain tumor in adults. Despite the highest mean years of potential life lost amongst all human cancers [5], the standard of care has not improved over the last two decades. Therefore, our primary focus in this narrative review will be on the complexity of GBM treatment, which will then be compared to other malignant gliomas in terms of heterogeneity, development, and treatment upon progression or relapse.

GBM has a diverse TME, consisting of heterogeneous and interactive populations of cancer cells and cancer stem-like cells, along with recruited stromal cell types, transformed parenchyma and associated stroma [3]. As well as the tumor cells, the TME includes the following different cell populations: (1) endothelial cells, (2) cells of lymphoid origin like effector T cells, regulatory T cells (Tregs), B cells and Natural Killer (NK) cells, (3) mesenchymal cells, (4) immune cells of myeloid origin, such as tumor-associated macrophages and microglia (TAMs, also known as glioma-associated macrophages or microglia, or GAMs), M2 macrophages, myeloid-derived suppressor cells (MDSC), tumor-associated neutrophils (TAN), and (5) fibroblasts [6]. Unique to the GBM environment are the neurons, glia, and microglia. Tumor cells communicate continuously with each other and with the TME via different chemical and electrochemical stimuli. The tumor can reprogram the function of the TME, resulting in an immunosuppressive and inflammatory environment in which cancer can thrive. The blood–brain barrier (BBB) limits the outflow of circulating tumor cells and thus aids in inhibiting extracranial metastasis, though this also complicates tumor analysis via non-invasive methods and limits treatment influx via systemic administration. The uniqueness of the CNS TME might further limit extracranial metastases to develop.

Here, we first outline the current Standard-of-Care (SoC) and tumor monitoring towards GBM treatment. Then, we zoom in on the most relevant GBM cancer-associated hallmarks, especially those implicated in GBM development, their spatiotemporal evolution, and their interplay with each other and the surrounding TME. These five hallmarks are deemed vital to understanding treatment mechanisms, all have currently available treatment options with repurposed drugs and are focus of current clinical research. We then compare adult and pediatric-type diffuse high-grade gliomas (diffuse pediatric-type high-grade glioma H3-wildtype and IDH-wildtype, diffuse midline glioma H3 K27-altered, and diffuse hemispheric glioma H3G34-mutant) to describe necessary treatment adaptations. Finally, we outline an empirically developed multiphase individualized combination treatment strategy to combat malignant glioma.

2. THE INFLUENCE OF THE GBM TME ON PROGNOSIS

Currently, GBM is treated with standard of care (SoC): maximal safe resection, radiochemotherapy (RCT) and, in most cases, maintenance chemotherapy (CTx) [7,8], though GBM displays high treatment-resistance [9].

GBM tumor cells show plasticity and dynamic changes in response to tumor development and treatment, which are in part influenced by epigenetic pathways [10]. As the tumor changes and evolves over time, the TME composition shows a similar spatio-temporal heterogeneity and adaptation. TME cells, such as myeloid cells, are interwoven throughout the tumor. Moreover, intra- and extratumoral communication paths exist through the formation of heterogeneous and cooperative tumor functional networks which contribute to tumor growth and therapy resistance [11,12]. Many GBM cells build ultra-long membrane protrusions, or tumor microtubes: direct, long-

distance (>500 μm) cell-to-cell connections used for brain invasion, proliferation, and therapy resistance. Cells interconnected via tumor microtubes are shown to be protected from cell death by RT. When damage to the tumor occurs, tumor microtubes are used for repair [13]. These interconnections occur through the formation of tumor microtubes [13], through autocrine/paracrine signaling, the release and uptake of exosomes [14], as well as through glioma-neuronal active synaptic activity [15–17].

Rather than one dense mass of tumor, different strictly regulated tumoral developmental areas exist. A tumor functions as a well-oiled machine, similar to a complex organ, a fact often disregarded both in clinical treatment as well as in general discussions [6]. The tumor and TME heterogeneity lead to strong patient-specific differences in anticancer treatment response. As different treatments directly affect the TME, new layers of patient-specific response and heterogeneity do not just develop, but are introduced [18]. Individual therapy response due to tissue heterogeneity harshly complicates randomized controlled trials designed with fixed treatment protocols, especially when focused on TME interventions, such as immunotherapeutic approaches [19]. Particularly here, the evaluation of the potency of a fixed treatment protocol (i.e., controlled conditions) might be affected by the patient-specific individual (i.e., uncontrolled) evolutionary dynamics of both the tumor and its TME.

Modern methods, such as DNA, RNA single cell sequencing (sc-seq), methylation profiling and micro-RNA (miRNA) profiling, have made great strides in clarifying tumor and TME development and heterogeneity, making it more possible to pinpoint which patient or tumor characteristics affect response to treatment, and which can be expected to respond. TME immune cell infiltration clusters (GTMEI) have been defined, describing genomic characteristics, molecular subtypes and clinicopathological features as well as proteomic, phosphoproteomic, acetylotomic, lipidomic and metabolomic properties [20]. To gain insight into the TME landscape of GBM, Zhao and colleagues assessed expression data of 25 immune cell types from 796 GBM patients. Data could be categorized according to miRNA expression (mi1 to mi5), DNA methylation profiles (dm1 to dm6), IDH status (mutant, wild-type), Gender (female, male), multi-omics (IDH mutant and nmf1 to nmf3), MGMT promoter methylation status (methylated, not methylated), age (less or above 55y), immune profile (im1 to im4), pathologic subtype (classical, IDH mutant, mesenchymal, proneural), WHO grade (high, low) and GTMEI-score: the quantification of immune infiltration patterns in any individual GBM sample [20].

Treatment is standardized for all GBM subtypes and GBM tumor status: despite the fact that tumors can have a different cause and a different developmental trajectory. Similarly to how each person differs from one another, no two tumors are alike. Even within the same person, tumor subclones might develop completely differently depending on its TME. In a RNA sequencing analysis of 107 patients suffering from GBM IDHwt, two subgroups could be identified based on specific gene regulation patterns [21]. These responder subtypes were found to be cancer-cell intrinsic and influenced by the TME. This is only one example of how the heterogeneity of GBM subtypes can impact patient prognosis and warrant variations in treatment. A better understanding of GBM subtype heterogeneity and their developmental trajectory will have a strong impact on course of treatment in a patient-specific way. To this end, new clustering techniques are being developed [22,23]. The rise of personalized therapy makes this realistically possible [24–26].

When considering the TME, the relative composition of 24 different types of infiltrating immune cells, together with checkpoint gene expression levels of CD27, PDL1 and CTLA4, can predict GBM prognosis from patient datasets. Three immune subtypes can be distinguished [27]. In immune subtype M1, central memory T cell (Tcm), T follicular helper cell (Tfh) and B cell subpopulations were most enriched. In M2, the top three highest enrichments include Tcm cells, GAMs and Tfh cells. In M3, the top three highest enrichments were Tcm cells, GAMs and B cells. The M3 subtype correlates with the worst prognosis, with high levels of immunosuppression, phagocytosis, leukocyte migration, and TNF superfamily members and -receptors. The M2 subtype, with the best prognosis, exhibited high enrichment scores in CD8+ response, B cell activation, B cell receptor signaling pathway activity, and transcription factor upregulation.

Therapy resistance, heterogeneity, fast development, a complex collection of important tumor characteristics, and predictive immune subtypes based on TME-tumor interactions, all highlight the complexity of GBM, a highly regulated heterogeneous system with spatiotemporal fluctuations that affect treatment response. These characteristics should be considered for individualized treatment plans, which should be adapted according to key GBM tumor and TME changes over time.

Though all fourteen cancer hallmarks play a significant role in the development of GBM, we will outline five key features that not only play an essential role in GBM tumor development, but can be approached with currently available treatment strategies. Listed numerically (1–5) in the order in which they will be discussed in text, each hallmark is also indicated by their icon ID-number: (1) Cancer stem cells and Glioma stem cells (GSCs) (hallmark 14); (2) Hypoxia (hallmark 5); (3) Metabolic reprogramming (hallmark 7); (4) Immune suppression and inflammation (hallmark 9); and (5) Neuron-glioma interaction (hallmark 1). These features are dynamically inter-connected, strongly influenced by the TME, and heterogeneously spatio-temporally represented.

2.1. Glioma Stem-like Cells (GSCs)

Functional GBM heterogeneity translates to multiple subpopulations of cancer cells [28,29]. Co-operation between genotypically and phenotypically distinct subclones explain the high level of invasion and migration seen in GBM [11]. Based on molecular signature, response to therapy and patient survival, GBM can be subdivided into three categories: proneural, classical and mesenchymal GBM. Comparing the three, the latter is especially linked to a worse patient prognosis. However, recent studies using spatial scRNAseq analysis have indicated that influences from the TME orchestrate a spatial and temporal fluctuation between the three subtypes within one tumor [30–32]. Often, all three subtypes can be found, with one subtype highly represented. External influences can cause transdifferentiation, especially towards the mesenchymal GBM subtype [33]. This further complicates efficient treatment. This cellular heterogeneity originates from a small subpopulation of glioma stem-like cells (GSCs, tumor hallmark 14, Figure 2). Less than 1% of tumor cells display GSC-specific properties of self-renewal and multi-lineage differentiation into neurons, astrocytes, and oligodendrocytes. This occurs in three ways: (1) dedifferentiation or reversal of a not yet terminally differentiated to a proliferating form, (2) blocked differentiation, or (3) transdifferentiation, during which cells of a particular phenotype undergo a morphological change, regain their proliferative abilities, and become clearly recognizable as elements of another tissue [3,28]. These three mechanisms underlie the formation of primary tumors, the progression of malignant cell growth, and affect response to therapy. GSCs are inherently resistant to GBM

treatment [28,34,35]. They can enter a quiescent status and so escape targeted CTx, and RCT, enabling cancer recurrence. GSCs communicate with their direct TME by cell–cell interaction via tunneling nanotubes (TNTs), causing a TNT-mediated transfer of mitochondria and thereby the modification of the target cell energetic metabolism with increased OXPHOS and ATP production [28]. TNTs differ from tumor microtubes in size ($<1\ \mu\text{m}$ width and $30\ \mu\text{m}$ in length to $1.7\ \mu\text{m}$ in width and $>500\ \mu\text{m}$ in length, respectively) and life span (TNT: 60 min., TM: >200 days) [36]. The GSC slow metabolism, efficient DNA damage response, and high drug efflux by ABC transporters, further aid GSCs to survive CTx and RCT. Furthermore, RCT and CTx such as Temozolomide (TMZ) consistently increase the GSC pool by causing further genetic mutations and triggering dedifferentiation in non-GSCs. Due to GSC development, GBM recurs more aggressively than the original tumor. It is considered vital to eliminate GSCs for complete tumor eradication. Due to their resistance to SoC treatment, suggested alternatives target GSCs through their deregulated metabolism [28]. Antidepressant drugs such as imipramine, amitriptyline, fluoxetine, mirtazapine, agomelatine, and escitalopram, are prescribed in cancer therapy to combat the side-effects of CTx. However, they were found to silence the phenotypic signature of GSCs, consisting of high expressions of CD44, Ki67, Nestin, Sox1 and Sox2. Particularly imipramine and amitriptyline were reported to modulate GSC plasticity and partially reverse GBM malignancy. These effects were linked to TME conditions and hypoxic fluctuations [37].

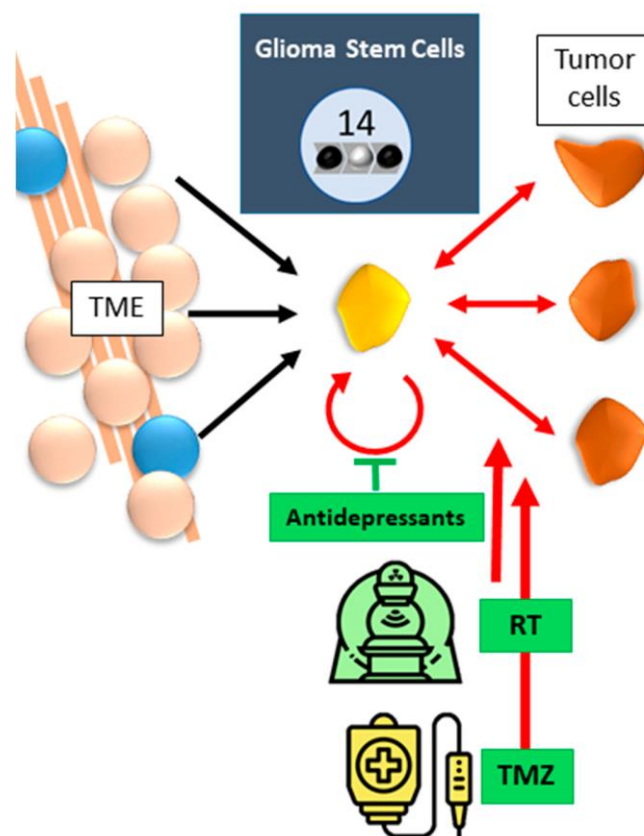


Figure 2. A simplified overview of the effect of glioma stem cells. GSCs can self-renew, differentiate and dedifferentiate to different GBM cell types (red arrows). This can be caused by RT or CTx (TMZ) treatment (red bold arrows). Antidepressants can inhibit GSC plasticity. GSCs rely on a permissive TME (black arrows.)

GSCs rely on a permissive specific TME niche. This niche consists of many different cell types, from stromal to immune cells, with many reciprocal communications essential for GSCs maintenance, survival and proliferation, as well as TME recruitment, activation programming, and persistence (Figure 2) [35]. Environmental factors originating from the TME affect the bidirectional plasticity of GSCs. For instance, hypoxia promotes a stem-like phenotype in non-GSCs, and the activation of epithelial-to-mesenchymal transition (EMT) further stimulates dedifferentiation of non-GSCs [35].

2.2. Hypoxia

Vascularization (tumor hallmark 5) normally occurs through a counterbalance of positive and negative signals, and consists of a complex interplay between soluble factors, membrane bound receptors, integrins and adhesion molecules. Tumors either interfere with regulatory integrins, or disrupt the balance between pro- and anti-angiogenesis signals. Balance disruption can occur through the overexpression of vascularization-stimulating factors, such as vascular endothelial growth factor (VEGF) through oncogene ras, or through downregulation of angiogenesis inhibitor factors such as thrombospondin-1 through loss of p53 function [1]. There exist six potential mechanisms of GBM vascularization: (1) Sprouting angiogenesis: the most common way of blood vessel formation via proliferation and migration of endothelial tip cells, (2) Vasculogenesis: Endothelial progenitor cells (EPCs) differentiate into new endothelial cells and form new vascular lumen, (3) Vessel mimicry: tumor cells form vessel-like channels, (4) Vessel Co-option: Tumor cells hijack existing blood vessels, (5) Cancer cell trans-differentiation: cancer stem cells differentiate into endothelial cells and form new vascular lumen, and (6) Intussusception: an existing blood vessel is split into two through mediated cell reorganization [29]. Through these mechanisms, GBM is capable of promoting tumor angiogenesis. However, this dysregulated process, combined with swift tumoral expansion, results in structurally and functionally abnormal vasculature, leading to areas of hypoxia [29,38–40]. Hypoxia-related vasculature plays a crucial role in tumor initiation and progression.

Within TME and tumor tissue, two types of hypoxia occur: chronic and cycling hypoxia (also called intermittent hypoxia) [41,42]. Chronic hypoxia is the first type to develop in tumors, and indicates a continuous ($\geq$ several hours) state of oxygen deficiency due to limited oxygen diffusion. Cycling hypoxia occurs due to inefficient perfusion in tumor blood vessels [43] and rapid changes in intracellular oxygen requirements due to metabolic changes. It marks fast fluxes between deep (<0.1 mmHg) and moderate hypoxia (15 mmHg). Both chronic and cyclic hypoxia occur with spatio-temporal fluctuation, forcing cells to adapt fast and collaborate for their needs. Cyclic hypoxia translates to a worse prognosis, as it affects many other cancer hallmarks: angiogenesis, intratumoral inflammation, immune evasion, swift cellular adaptation pushing tumor metastasis, intratumoral heterogeneity, and resistance to treatment. In addition to that, the reoxygenation periods give rise to reactive oxygen species (ROS) [41,42].

Hypoxia leads to SoC-resistance. During RCT, oxygen increases DNA damage through free radical formation; thus, anoxic cells require a radiation dose three times higher to obtain similar effects to treated oxygenated cells [41]. Under hypoxic conditions, metabolic pathways become so dysregulated in GSCs that tumor cells must fall back on autophagy to obtain energy. Autophagy also functions as a protective mechanism against CTx in GBM. Cycling hypoxic tumor cells isolated from GBM xenografts using flow cytometry exhibited higher ABCB1 expression and CTx resistance than chronic hypoxic cells and normoxic cells [41]. Hypoxia-inducible factor (HIF)-1 α is a common

driver of pathways that define GBM aggressiveness (Figure 3). Downregulation of HIF-1 α activity is associated with increased responsiveness to TMZ [38–42,44] and RCT [41,42]. The deregulation of HIF-1 α due to hypoxia can be combatted with Mebendazole, an antihelminthic benzimidazole [45]. GBM patients treated with chloroquine, an autophagy flux suppressant, display reduced chemoresistance and improved survival (Figure 3) [46]. According to clinical trials, the nontoxic hormone melatonin is an antiangiogenic, antiproliferative and metastasis-inhibitory agent that enhances the effectivity of other therapeutics when used in combination [47,48]. In GBM, melatonin was found to suppress the HIF- α /VEGF/MMP9 axis through miRNA regulation [49]. As a modulator of the circadian rhythm, melatonin is normally present in the blood and cerebrospinal fluid (CSF). Melatonin concentrations reach a maximum of 80 to 120 pg/mL between 02:00 and 04:00, and drop sharply to between 10 and 20 pg/mL during the day. These inherent temporal fluctuations affect doses and time of administration [48]. Bevacizumab, or Avastin, targets VEGF and specifically combats tumor angiogenesis [50].

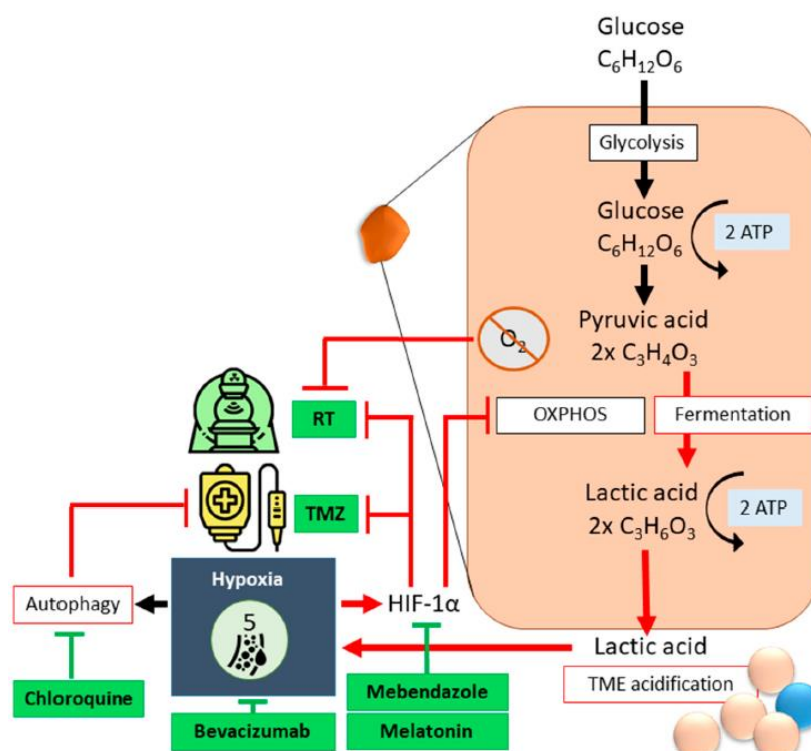


Figure 3. A simplified overview of the effects of hypoxia in GBM. GBM Effects are displayed with red arrows, treatment options are shown in bright green. Hypoxia normally leads to autophagy (black arrow) which inhibits the effects of RT and CTx such as TMZ. Chloroquine treatment can inhibit autophagy. Bevacizumab inhibits angiogenesis factor VEGF. Hypoxia also includes upregulation of HIF-1 α , which inhibits both CTx effects and the OXPHOS pathway, driving cellular metabolism toward anaerobic fermentation. Mebendazole and melatonin can be used to normalize HIF-1 α expression levels.

It was recently found that some malignant glioma cells can maintain a glycolytic metabolism despite their hypoxic environment by macrophage-mediated recycling of myelin cell debris [51]. Upon scavenging cholesterol-laden myeloid cell debris, GAMS develop into lipid-laden macrophages (LLMs). These LLMs are epigenetically rewired to display immunosuppressive features and are especially enriched in aggressive mesenchymal GBM. Through dynamics in

correlation with GBM stage, cellular subtype composition and local tumor niches, LLMs transfer their myelin cell debris cargo to cancer cells, fueling their tumorally increased metabolic demands [51]. This again clearly displays the closely controlled spatial intra-cellular interaction between TME and tumor, and depicts how the development of hypoxia within tumor tissue affects the cellular metabolic programming of both healthy and diseased tissue.

2.3. Metabolic Reprogramming

We will zoom in on three different levels of GBM metabolic reprogramming, which strongly affect the aforementioned hallmarks: energy generation, lipid metabolism and nucleotide metabolism (Figure 4) [52].

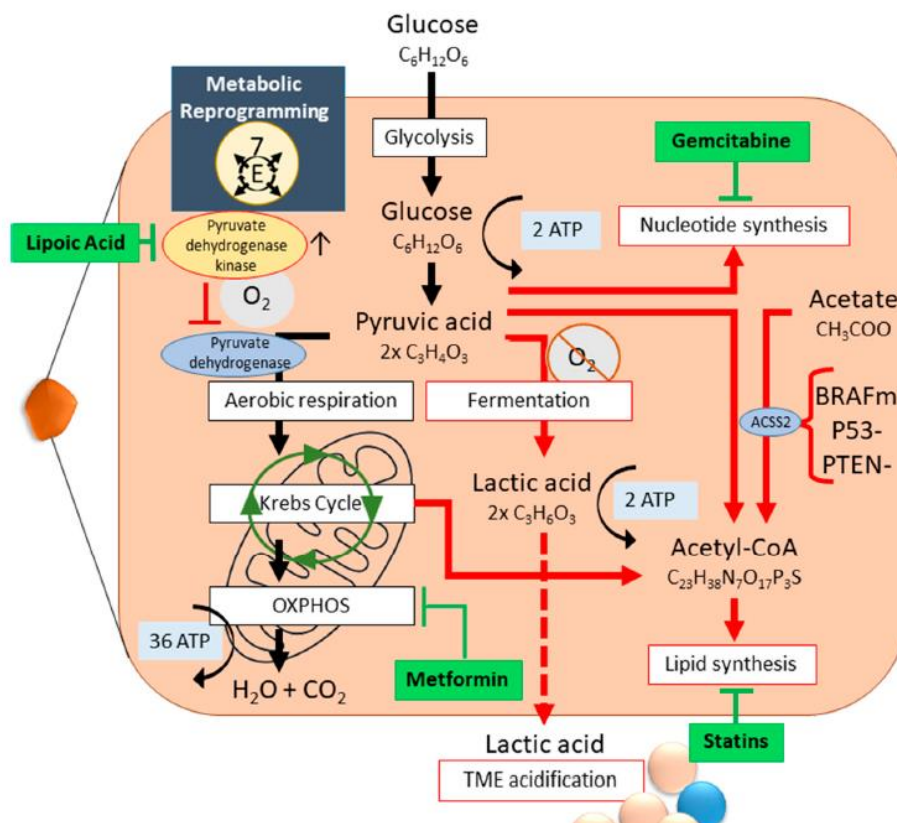


Figure 4. A simplified overview of metabolic reprogramming in GBM. Normal pathway steps are depicted with black arrows, GBM pathway steps are shown in red. Possible treatment options are depicted in bright green. Glucose is processed through glycolysis to intermediates, ATP and NADH. In cancer cells, an increase in pyruvate dehydrogenase kinase (PDK) inhibits aerobic respiration, favoring an anaerobic fermentation pathway instead. Lactic acid production leads to the acidification of the TME. Treatment with lipoic acid inhibits PDK. Metformin can then be used to inhibit the OXPHOS pathway in all fast-growing cells, specifically targeting tumor cells. Combining TMZ with Metformin treatment can revert chemoresistance.

Under normoxic conditions, healthy cells process glucose through glycolysis and aerobic respiration to pyruvate and subsequently to CO_2 , generating 38 ATP. In tumor cells, including GBM, the glucose, glutamine and fatty acid metabolism are heavily altered [34] (Hallmark 7, Figure 4). Due to increased proliferation, tumor cells opt for aerobic glycolysis, known as the Warburg effect [53,54]. The intermediary glycolysis products are used for cellular growth, inhibiting aerobic

respiration [34]. Lack of oxygen and redirection of necessary intermediary products pushes anaerobic fermentation, generating only four ATP [34]. Metabolic reprogramming leads to the development of different intratumoral subpopulations which collaborate to ensure an all-round sufficient nutrient supply, ROS clean-up, and energy (Figure 4) [39,40]. As a result of metabolic reprogramming and hypoxia, the electron transport chain collapses, leaving the cell with acute ATP depletion. Areas of necrosis develop within the tumor [55]. Even without hypoxia, necroptotic areas occur. Necrosis in GBM is a well-regulated malignant process: it involves neutrophil-triggered ferroptosis; accumulation of neutrophils within GBM correlates both temporally and spatially with necrotic areas. Neutrophil depletion dampens necrotic area occurrence [56]. GBM necrosis induces massive angiogenesis through VEGF expression and releases high mobility growth factor (HMGB-1), promoting tumor proliferation and invasion by elevated IL-8 expression in peri-necrotic regions [55,57]. Cytosolic proteins released during necrosis elicit a pro-inflammatory cytokine response, which aid in angiogenesis, GBM migration, and chemo-resistance [55]. Necrotic foci in GBM are considered a pathological and radiologic hallmark, signaling an overall poor prognosis. GBM patients whose cells do employ the OXPHOS pathway have a better prognosis [52,58]. OXPHOS exclusion is enriched in the mesenchymal GBM subtype, and is, as noted before, linked to SoC resistance.

Lipids are fat-soluble organic compounds with structural, signaling and storage functions. The TME of GBM is a lipid-rich environment, as the brain dry-weight naturally exists for 50% of lipids. The side-products of glycolysis and the Krebs cycle, normally used for energy generation, are redirected for cytosolic acetyl-CoA synthesis. Simultaneously, extracellular acetate import is upregulated and used to produce acetyl-CoA via acetyl-CoA synthase (ACSS2), which is upregulated through BRAF, p53 and PTEN mutations and malfunction, common in GBM [52]. GBM requires elevated levels of de novo lipogenesis for proliferation, and not only as a structural component: GSCs rely heavily on fatty acid oxidation. Thus, the aberrant lipid metabolism is heterogeneous throughout tumors, and combination therapies are advised. The lipid metabolism, including the fatty acid oxidation and de novo lipogenesis, is a potential target for statins (Figure 4) [52].

As important signaling and structural components, nucleotide production is highly upregulated in GBM. Nucleotides can be synthesized de novo or via salvage pathways: GBM relies heavily on the former, which is orchestrated by c-MYC activity. However, salvage pathways are also used. The ability of GBM cells to scavenge hypoxanthine, the most abundant purine present in the CSF, is thought to cause GBM anti-folate therapy resistance [52]. Gemcitabine is a cytidine analog which, when incorporated into the DNA, inhibits ribonucleotide reductase. Gemcitabine can cross the BBB and accumulates in GBM [59].

As the source of nutrients and oxygen, the TME plays a major role in GBM metabolic reprogramming. For instance, hypoxia activates aerobic glycolysis and triggers EMT through HIF. Metabolic reprogramming and release of ROS accelerate TME acidification. GSCs are dependent on strong metabolic reprogramming, mitigated by the TME [33,35]. Metformin combats cancer cells in three distinct ways, effectively targeting both non-GSCs and GSCs through their aberrant metabolism [35,60]. Firstly, metformin rewires the metabolic function from anaerobic fermentation back to glycolytic. Transcriptional analysis of macrophages derived from metformin-treated mouse bone marrow cells show a decreased expression of pro-tumor genes such as transforming growth factor (TGF)- β and interleukin (IL)-1 β [60]. Secondly, metformin targets tumor cells that use

glycolysis by blocking glucose uptake and inhibiting complex I of the mitochondrial electron transport chain to disrupt oxidative phosphorylation (OXPHOS) and subsequent mitochondrial ATP generation. The lower glycaemia, lower glucose uptake, and lower ATP levels in tumor cells, also due to hypoxia, inhibit mTOR complex 1, a major hub for cell growth, protein translation and cellular metabolism. Thirdly, metformin plus TMZ can revert chemoresistance under hypoxic conditions via PI3K/mTOR pathway suppression (Figure 4) [61].

2.4. Immune Suppression and Inflammation

Cell-to-cell TME and GBM interactions, metabolic alterations, spatio-temporal oxygen fluctuations, and cellular necrosis together create a constant state of inflammation within the TME (hallmark 9, Figure 5). Rather than help combat the tumor, this perpetual inflammatory environment enhances tumorigenesis and progression by promoting other cancer hallmarks. Pro-inflammatory biomolecules drawn to the inflamed TME include: (1) growth factors that sustain proliferative signaling; (2) survival factors that inhibit apoptosis; (3) proangiogenic factors that nurture vascularization, which in turn promotes invasion and metastasis; and (4) inductive signals that lead to EMT activation. Multiple occurrences are known in which a perpetuating inflammation instigated tumor formation. A vicious circle develops, resulting in an immunosuppressive TME [41], supporting critical immune components such as MDSCs and GAMs [58,62–65]. The alteration of glycans and lectins further promotes tumor progression and immune evasion by enhancing immunosuppressive cell subsets while impairing immune effector populations. These pathways, along with their associated receptors (C-type lectins, Siglecs, and galectins), represent promising therapeutic targets. [66]

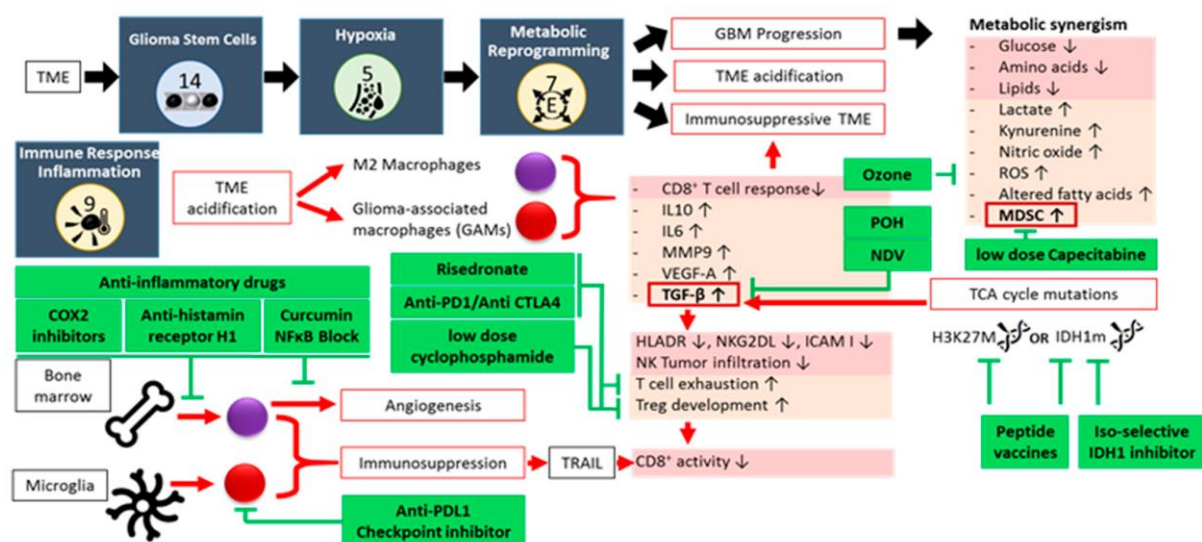


Figure 5. A simplified overview of the effects of immune response inflammation in GBM. Tumorspecific occurrences are with red green. Downregulation (downward black arrows) of cell types or cytokines is highlighted in red, and upregulation (upward black arrows) is highlighted in orange. Due to TME acidification, M2 Macrophages and GAMs are upregulated, which inhibits cytotoxic T cell response but increases inflammation-regulating cytokines, developing into an immunosuppressive TME. Especially the upregulation of TGF-β, by both tumor-associated macrophages as well as TCA cycle mutations, further inhibits the innate and adaptive immune system. TCA cycle mutations can be halted with ONC201 treatment, isoselective inhibitors and peptide vaccines. GAMs, especially when derived from bone marrow, also aid angiogenesis. PDL1+

M2 Macrophages target CD8+ T cells via the apoptotic TRAIL pathway, both during CD8+ T cell priming and effector function. Using anti-PDL1 checkpoint inhibitors, this PD1-independent immunosuppression can be inhibited.

The interplay between GBM and MDSCs results in a metabolic synergism, which causes further deprivation of glucose, amino acids and lipids, as well as accumulation of lactate, kynurenine, nitric oxide (NO), ROS, and altered fatty acids. This stimulates further MDSC recruitment and activation, immunosuppression, reduction in T cell responsiveness, and TME acidification [67]. Under acidic conditions, macrophages are polarized to the M2 phenotype [68]. A recently discovered immune suppression mechanism involves PERK-driven glucose metabolism in M2 macrophages. Through glycolysis-dependent histone lactylation, a recently identified post-translational modification, a IL-10 expression and a T-cell suppression are promoted in GBM [69]. Lactylation regulates gene transcription and is closely linked to the Warburg effect, with lactate as the key driver in the TME. This modification occurs on histone and non-histone proteins, including oncoproteins, and is implicated in various physiological and pathological processes [70]. The continuous low-dose, or metronomic, administration of capecitabine, an orally available 5-fluorouracil (FU) CTx prodrug, reduces circulating MDSCs and increases cytotoxic immune cell tumor infiltration [71]. Ozone therapy can further reduce ROS [72].

Microglia/macrophage functions are almost immediately altered upon exposure to the GBM TME secretome, and give rise to GAMs [64], which make up about 50% of total live GBM biomass [73]. GAMs in GBM further contribute to the construction of the immunosuppressive TME by expressing high levels of IL-10, IL-6, TGF- β , angiogenic molecules (VEGF-A), matrix metalloproteinases (MMP9), and by inhibiting CD8+T cell infiltration [74,75]. GAMs originate from two independent sources: bone marrow (85% in GBM) and microglia (15% in GBM). Each subtype performs different functions at different stages of tumor progression [18,76,77]. Though macrophages and microglia in GBM have morphological and behavioral changes, it was deemed problematic to distinguish between the two. Advanced methods including scRNAseq, cellular indexing of transcriptomes and epitomes by sequencing (CITE-seq), and cytometry by time of flight (CyTOF) have shown that macrophages show enriched levels of CCR2, CD45RA, CD141, ICAM, CD1C, CD1B, TGFBI, FXRD5, FCGR2B, CLEC12A, CLEC10A, CD207, CD49D, and CD209, whereas microglia express high levels of CX3CR1, SALL1, HEXB, P2RY12, and TMEM119 [73,78]. Primary GBM is more likely to be infiltrated by GAMs derived from microglia, whereas they are absent from the core of metastatic brain tumors, which are mainly inhabited by bone marrow-derived GAMs [73]. The polarization of microglia or bone marrow-derived macrophages has been suggested to mediate the immunostimulatory effects of certain treatments, including RT. The more dynamic M2 macrophages increase tumor growth and suppress immune responses, whereas the more-defined M1 subtypes reduce tumor growth, improving RT efficacy [18]. The bone marrow-derived macrophage population exerts immunosuppressive and proangiogenic effects. Anti-inflammatory drugs like COX2 inhibition [79], anti-histamine receptor H1 drugs [80] and curcumin [78] can influence their biological role. Microglia are unique to tumors in the CNS. These main resident myeloid cells play a crucial role in the surveillance, development and maintenance of the brain [64,65,73]. They are involved in neurogenesis and axonal growth, and orchestrate immune responses against pathogens or damaged cells. Microglia exhibit a broad functional diversity and can be classified into multiple subtypes based on their characteristics, which depend on the brain developmental stage and tissue location [81,82]. They switch between different functional states depending on microenvironmental cues. (Figure 5).

Due to their aberrant expression patterns, GAMs can be targeted for treatment, though thus far with limited success [73]. GAMs overexpress PDL1, TNF and TNF-related apoptosis inducing ligand (TRAIL), but not Fas ligand [83]. Of particular interest is the finding that PDL1-expressing GAMs block CD8⁺ T cell responses (priming and effector function) via TRAIL signaling [83]. This suggests that targeting GAMs via PDL1 can rescue CD8 T cell responses upon dendritic cell vaccinations.

The resident T cell population in the TME is heavily altered by tumor development. Cytotoxic activity is downregulated by promoting T cell exhaustion, and by an increased presence of Tregs. Checkpoint inhibitors targeting T cell surface markers, such as Anti-PD1 or Anti-CTLA4, inhibit cytotoxic T cell exhaustion by tumor cells [84]. Nitrogen-containing bisphosphonates, such as risedronate, have a direct anti-proliferative effect on tumor cells through Ras and Rab modification [85], but also reactivates cytotoxic T cell activity [86]. Monoterpene and Ras inhibitor perillyl alcohol (POH) have been shown to significantly increase overall survival in GBM patients, independent of their MGMT status, by inhibiting the cell cycle. They also act through TGF- β , the endoplasmic reticulum (ER) stress pathway, and by arresting survival pathways such as mTOR. When administered intranasally, POH crosses the BBB and thus affects GBM tumors which are otherwise difficult to reach [87,88]. Cyclophosphamide (CPM) is an alkylating CTx agent. Similarly to risedronate, CPM has direct cytotoxic and immune-modulatory effects. Low dose CPM has been shown to deplete regulatory T cells [89] and increase CD4⁺ and CD8⁺ T cells in the TME, as CPM downregulates TGF- β receptor 2 expression levels [90,91]. It was found most effective as a metronomic administration, resulting in a reduced myofibroblast population [91,92].

A critical link between the aberrant glycolysis and immunosuppression is the production of TGF- β [63]. TGF- β down-regulates tumor surface antigens such as HLA-DR and NKG2DL, and intercellular adhesion molecule I. Infiltration of NK cells in early stage of tumorigenesis is inhibited by TGF- β . TGF- β plays a role in T cell exhaustion. Through TGF- β signaling, CD4⁺CD25⁻ naïve T cells develop into T-regs that inhibit tumor-specific CD8⁺ T cell cytotoxicity and NK cell function. However, aberrant TGF- β production also weakens the tumor cell immune system, allowing oncolytic viruses otherwise unable to infect human cells, such as Newcastle Disease Virus (NDV), to specifically infect and kill the tumor from the inside out [93,94]. Immune function is further hampered by mutations in TCA cycle-enzymes. The accumulation of the oncometabolite D-2-hydroxyglutarate in IDH-mutated gliomas contributes to the “cold” tumor immune phenotype [95]. The mutation of IDH1 suppresses STAT1, a regulator of CXCL10, leading to reduced CD8⁺ T cell accumulation in gliomas [96]. The “cold” tumor immune phenotype might shape the TME, allowing the development of tumor subclones with higher-grade features. Isoformselective IDH inhibitors and peptide vaccines targeting IDH-mutant gliomas have shown translational potential in preclinical and clinical studies by interfering with both the cellular metabolism and the relative composition of target-specific tumor-infiltrating cytotoxic T cells [97,98]. Overall, the aberrant metabolism creates metabolic checkpoints underlying the immunosuppressive TME [99].

2.5. Neuron-Glioma Interaction

Neoplastic cells elicit sustained proliferative signaling (hallmark 1) in three ways: (i) autocrine signaling; (ii) overexpression or structural alteration of membrane-bound growth signal transducers; and (iii) disruption of intracellular matrix receptors (integrins), favoring transmission of proliferative signals [100]. The latter occurs in at least 30% of tumors, including melanoma, breast cancer, ovarian cancer, colon cancer, thyroid cancer, prostate cancer, and glioblastoma [100,101].

Not considering certain TME and tumor-specific aspects, the key features discussed up to now play a role in any type of tumor. However, there is one key feature truly unique to GBM that bears consideration: the neuron-glioma axis (Figure 6) [15,16,73,102–105]. Neuronal activity drives tumor progression through paracrine signaling factors such as neuroligin-3 and brain-derived neurotrophic factor [1–3]. A direct glutamatergic synaptic input from presynaptic neurons to postsynaptic glioma cells sustains the glioma cell network and drives glioma progression [16,105]. Postsynaptic currents are mediated by glutamate receptors of the AMPA subtype, which can be targeted with the AMPA receptor antagonist peramppanel (Figure [16]. Levetiracetam can target the GABAergic synaptic communication node [106]. Direct neuro-immune interactions occur via GABA-associated immune cell subtypes like M2 macrophages and T cells, contributing to tumor immunosuppressive mechanisms (Figure 6) [107]. Knowledge in the domain of cancer neuroscience is growing fast, as several cancer hallmarks intersect with neuroscience in the CNS TME [108].

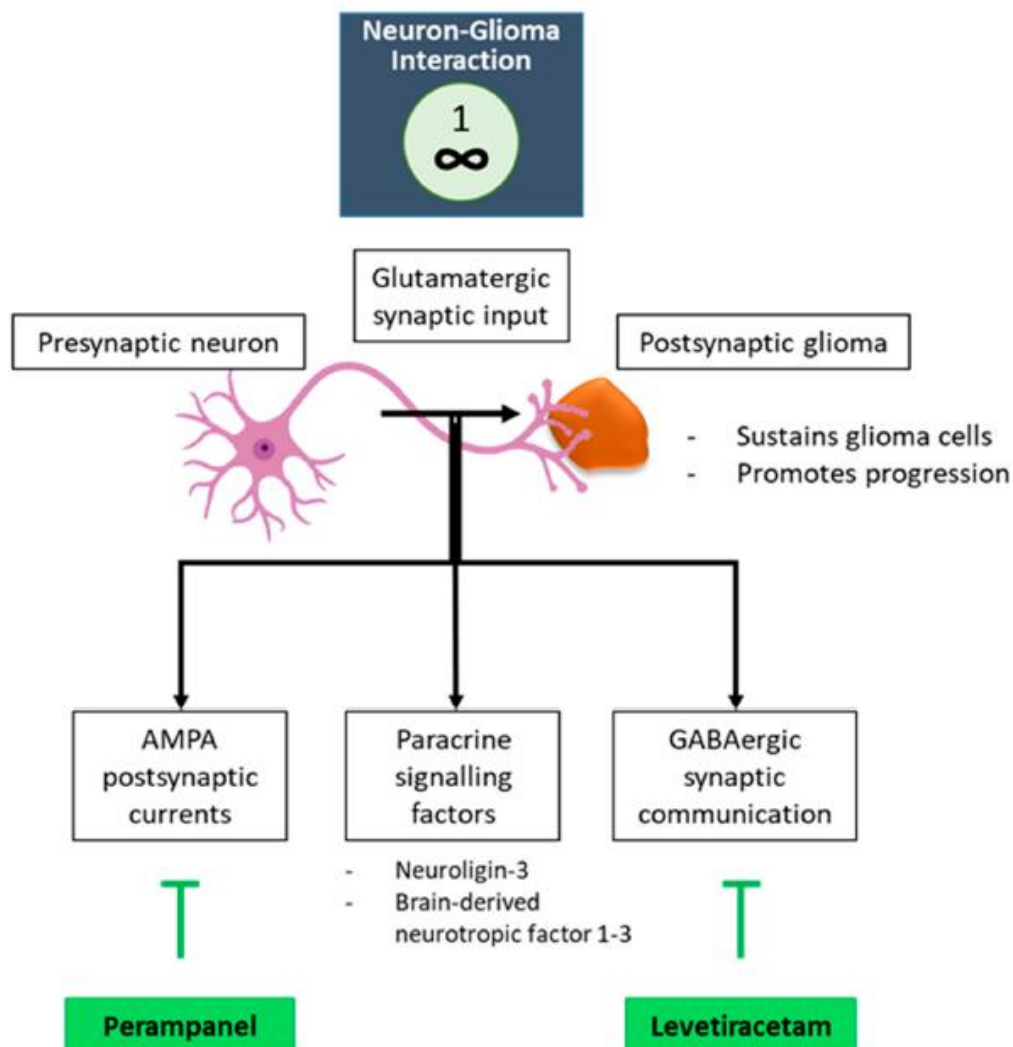


Figure 6. A simplified overview of the effects of the neuron-glioma interactions. The interactions between presynaptic neurons and postsynaptic gliomas drive tumor development through AMPA postsynaptic currents, paracrine signaling factors, or GABAergic synaptic communication. The first can be inhibited with peramppanel and the last one with levetiracetam.

3. GBM VERSUS PEDIATRIC-TYPE DIFFUSE HIGH-GRADE GLIOMAS

Brain tumors are the leading cause of cancer-related child mortality [109]. Diffuse midline gliomas (DMG) are considered one of the most aggressive pediatric brain malignancies. DMGs, also known as diffuse intrinsic pontine glioma (DIPG) when located in the pons, typically affect children between the age of 5 and 10, but also occur in adolescents and adults. DIPG is a uniformly fatal diagnosis, with a median survival of less than one year, due to its location in the brainstem which makes resection impossible. Over a hundred clinical trials and testing methods such as radiosensitizing agents, cytotoxic or high-dose chemotherapy with or without stem cell rescue, or molecularly targeted agents, showed no OS improvement over RT. Even if a child makes it through harsh rounds of treatment, the damage done to the developing brain results in much more severe loss of quality of life than adults who undergo the same SoC. Often, pediatric treatment is thus focused on minimizing toxicity [110]. Due to the many challenges in studying pediatric brain malignancies, progress in understanding tumor development and immunotherapy is slow [81]. Many pediatric brain tumors are thought to arise during prenatal brain development as embryonal tumors, and coincide with periods where unique subpopulations of immature microglia are abundant, which are therefore implicated in pediatric malignancies [81,110]. Although some childhood brain tumors share similarities with adult GBM, many are distinct entities, residing in locations different from adult brain tumors, and thus have a completely different TME. Mechanistic characteristics and treatment methods can be compared across adult-type diffuse glioma, particularly IDH-wildtype GBM, and diffuse pediatric high-grade gliomas. We will specifically elaborate on diffuse pediatric-type high-grade glioma (pDHGG) H3 wild-type and IDH1 wild-type, diffuse hemispheric glioma H3G34-mutant (H3 G34-mutant DHG), and diffuse midline glioma H3K27-altered (H3 K27-altered DMG) [4,111].

DHG), and diffuse midline glioma H3K27-altered (H3 K27-altered DMG) [4,111]. Similarly to adult GBM, pediatric-type diffuse high-grade gliomas are highly heterogeneous [112]. The immune and inflammatory cellular players in the TME are quite similar to the adult counterpart; the major immune infiltrates are macrophages and T cells, though they are extremely sparsely available [77,113]. It was suggested that as the tumor develops from low to high grade malignancy, a transient change in the relative composition of T cells and myeloid cells occurs: effector T cells make room for Tregs, microglial cells diminish, GAMs increase and give rise to a large population of monocyte-derived M2 macrophages. The TME in pediatric-type diffuse high-grade gliomas is characterized by hypoxic regions and low nutrients, similar to the GBM TME, which translates to an immunosuppressive environment. However, there exist significant general and specific differences between pediatric and adult malignant gliomas, both on the genetic and epigenetic level [77,81,113]. As the CNS develops, a diversity of unique immature microglia is abundantly present, which presumably not only contributes to tumor onset and progression, but results in a glioma characterized by a relatively high number of microglia with aberrant morphologies and phenotypes. This suggests a crucial role in TME development [77,81]. Complications and treatment options connected to glioma-neuron interactions therefore carry more weight in pediatric-type diffuse high-grade gliomas, in comparison to adult GBM. Pediatric tumors need more frequent monitoring, both to balance out the faster change in a developing brain compared to a fully developed, adult brain, but also as this “young” environment is more prone to GSC occurrence. Children with brain tumors are thus more prone to develop radiotherapy resistance, and are simultaneously more vulnerable to RTs neurotoxic effects [110,114]. Immunotherapeutics are considered an answer to this toxicity problem: their ability to target the tumor specifically minimizes organ damage and allows

for tumor treatment despite its inaccessible location. This, however, comes with complications: adult clinical trial results cannot be used for pediatric patients, as their immune system is not yet fully developed. Compared to adult GBM, pediatric malignancies have a low mutational burden, which means checkpoint blockade strategies are less effective. Adding to this is the fact that immunotherapy development has often been carried out through research on adult GBM in immunocompromised mice. Only scant evidence exists on immunotherapies in children, due to ethical objections and fast disease progression [110].

The diffuse pediatric-type high-grade glioma (pDHGG) (H3-wildtype and IDH-wildtype), historically known as pediatric GBM, shares a similar immune response to adult GBM based on its immune infiltration profile [81]. Both have an increased antiinflammatory myeloid cell population with high M2 and PD1 marker expression and a reduced CD8+ T cell and NK activation rate. Both contain necrotic areas. Spatially resolved single-cell analysis of diffuse pDHGG (H3-wildtype and IDH-wildtype) have indicated adenosine-rich regions harboring specific immunomodulatory functions involving microglia. The spatial distribution of immune cells within the TME is considered crucial for understanding and identifying targetable vulnerabilities in pDHGG (H3-wildtype and IDH-wildtype) [81].

The immune cell composition is different in H3 G34-mutant DHG. Most tumors belong to a pediatric high-grade glioma immune cluster type 3, with moderate levels of CD8+ T cells and relatively low levels of other infiltrating immune cell types [113]. Curiously, a higher than median concentration of CD4+ T cells and NK cells was associated with poor survival. Another study characterized H3 G34-mutant DHG as a tumor with a higher level of immune cell infiltration, with higher levels of monocytes and CD4+ T cells, but lower Treg infiltration [77].

Mutations of IDH1 and H3 K27M are mutually exclusive, resulting in a completely different phenotype in H3 K27-altered DMGs compared to their IDH1-mutant counterparts, but also when compared to pDHGG (H3-wildtype and IDH-wildtype) and adult GBM [77,115,116]. DMG tumors are generally found to have a comparatively high inflammatory, “cold” TME characterized by a low T cell infiltration. DMG is typically known for a high infiltration of macrophages or microglia, but limited to no T or NK cells, and an absence of chemokine or cytokine expression to recruit these lymphocytes. This does not mean the present microglia are completely inactive: DMG cells can induce a loss of histone 3 lysine 27 trimethylation in microglia and consequently affect its phenotype to a tumor-promoting state, contributing to the highly immunosuppressive conditions within DMGs [81]. However, it was shown that DMG had a greater inflammatory TME compared to the hemispheric high-grade gliomas, including hemispheric pDHGG, and displayed a greater expression of immune-related genes indicating the presence of T cells, macrophages, neutrophils and CD45+ cells [117]. The understanding of the biological function of inflammatory cells in DMG progressed fast with the development of highly innovative syngeneic allograft mouse models, of which the histopathology, the immune microenvironment and the therapeutic response of DMG adequately recapitulates human DMG [118]. Of particular interest is the high PDL1 expression in tumor-associated versus healthy microglia and macrophages, demonstrated at both the single-cell RNA and protein level using CITEseq analysis of syngeneic DMG tissues (Figure 7). As previously noted, PDL1 overexpression in GAMs as compared to non-tumor-associated macrophages is prevalent in DMG (Figure 7). GAMs driving T cells from a progenitor exhaustion state to terminal exhaustion has also been described [119].

These data are confirmed by findings in human DMG tissue (communication of unpublished data by DSM and EH). A recent review assessed MDSCs as a potential immunotherapy target in DMG [120]. Overall, these data form the rationale for targeting the myeloid component in DMG treatment. Metabolic alterations in H3 K27-altered DMG cells include an increased glucose dependence for energy with subsequent elevated lactate production levels, an increased cholesterol biosynthesis, increased TCA and OXPHOS metabolism, increased glutaminolysis and increased Pentose Phosphate Pathway activity [121]. An observed increase in DMG citrate concentration (also seen during hypoxia) combined with the reduced relative tumor blood volume detected by advanced magnetic resonance spectroscopy (MRS) and -imaging (MRI), and their improvement upon treatment, strongly suggest that intratumoral hypoxia also plays a prominent role in DMG [122]. GABAergic synaptic communication between GABAergic interneurons and DMG cells were discovered, underscoring tumor subtype-specific mechanisms of brain cancer neurophysiology [106], and suggesting the potential need for the additional blockage of the GABAergic pathway with levetiracetam besides blocking the glutamatergic pathway with perampanel in DIPG [123]. One particular type of dopamine antagonist drug, the first in line being ONC201, emerged recently as promising against DIPG/DMG. ONC201, or dordaviprone, selectively binds dopamine receptor D2 (DRD2) and so inhibits cell proliferation via the Ras pathway [124]. It also binds to mitochondrial caseinolytic protease P (ClpP) which impairs mitochondrial activity and subsequent apoptosis [125]. Though ONC201 treatment does not prolong overall survival of malignant glioma in general, ONC201 in H3 K27-altered DMG does have a positive effect. H3 K27-altered cells use α -ketoglutaric acid (α -KG) to maintain a low methylation status (H3 K27me3). The application of ONC201 increases H3 K27me3 by increasing glutamine over glucose derivation of α -KG, which is then metabolized to 2-hydroxyglutarat instead [124]. As ONC201 activates the ATF4/CHOP-mediated integrated stress response via downstream target engagement, it indirectly activates the TRAIL/Death receptor 5, inactivates Akt/ERK signaling in tumor cells, and inhibits OXPHOS via c-myc [126]. TRAIL-independent apoptosis, cell cycle arrest, and antiproliferative effects also occur. As this extends the effects of ONC201 beyond tumor bulk to include cancer stem cells, associated fibroblasts and TME immune cells, it is unsurprising that clinical studies have shown that ONC201 does not only have a clinical activity on its own but also synergizes with RT, CTx, targeted therapy and immune checkpoint agents [126].

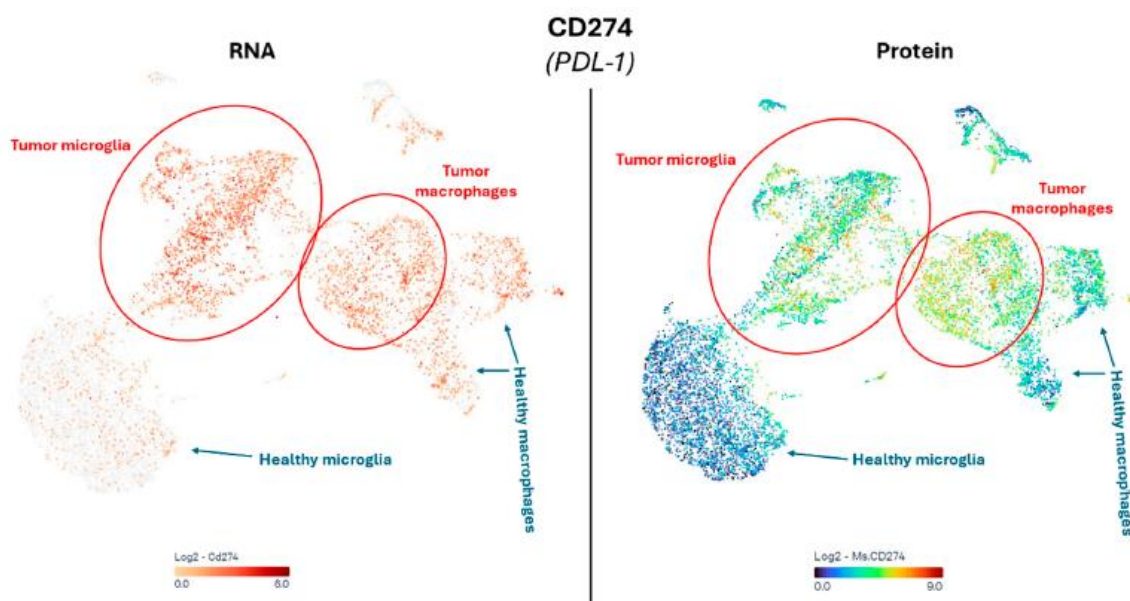


Figure 7. Single-cell RNA and protein analysis of myeloid cells in the pontine region of syngeneic allograft mouse models for DMG, indicating PDL1 expression in GAMs compared to healthy cells. Color scales indicate expression levels. Red circles indicate the different tumor-associated grouped cells.

4. NEED FOR INNOVATIVE MULTIPHASE INDIVIDUALIZED COMBINATION TREATMENT

Though each key feature can be linked to a specific tumor hallmark, all of them are in fact the result of complex interplay between multiple tumor hallmarks, and closely interact and affect one another. GSCs communicate with their direct TME by cell-cell interaction via TNT, directly affecting the energetic metabolism of their surrounding cells [28]. The oncogenes, which cause malignancy, often directly deregulate the cellular metabolism: TP53 regulates the lipid metabolism in cancer cells, and c-MYC activates glutamine uptake [34]. Glutamine competition halts immune cell activation, and inhibits CD4+ T cell development into inflammatory subtypes. Intermediary and end products of glycolysis and lactate fermentation inhibit NK cell function, Th1 differentiation, B cell function and T cell activation. Competitive glucose uptake is the main cause of impaired T cell function in the TME [127]. The regulation of vasculature and angiogenesis results in the formation of fluctuating areas of hypoxia and varying nutrient availability. This, in turn, contributes to the deregulation of metabolism, and non-mutational epigenetic reprogramming, promotes tumor tissue heterogeneity, and increase invasiveness and metastasis [41,75]. Oxygen concentrations play a fundamental role in stemness maintenance, defining several GSC niches. Hypoxia even enlarges the GSC population [41]. One can distinguish five hypoxia-related niches [29]: (1) the perivascular niche where proneural GSCs mainly reside; (2) the immune niche; (3) the hypoxia/necrotic niche where mesenchymal GSCs mainly reside; (4) the extracellular matrix niche; and (5) the peri-arteriolar niche. All of these niches also interact with each other.

Taken together, these subsequent changes and intratumoral fluctuations create a temporal effect within hallmark development. This explains the heterogeneity seen in tumors, as well as their fast-adapting character over time. An example of this is the fact that when fluctuating areas of hypoxia develop due to angiogenic deregulation and changes in metabolism, different cell populations develop within one tumor which collaborates to regulate nutrient availability throughout the tumor tissue [34]. It also explains why tumors can exhibit such different and fast-changing behaviors and differences in development between people, tumor locations, and over time [3,34,40,75]. Figure 8 outlines how, eventually, the different tumor hallmarks together can influence tumor development, and how repurposed drugs can target different hallmarks. A chance interplay between genomic mutations (hallmark 10) and non-mutational epigenetic reprogramming (hallmark 11), deregulates the cellular metabolism (hallmark 7), which in turn causes a cell to evade growth suppression (hallmark 2) and leads to subsequent cellular senescence (hallmark 13) as the cell proliferates. The genomic instability and fast cell division of the starting malignant cells lead to resistance to apoptosis (hallmark 3). The cells instead gain sustained proliferative signaling (hallmark 1) and maybe even replicative immortality (hallmark 4). Phenotypic plasticity is unlocked (hallmark 14), which not only allows the growing malignancy to avoid immune destruction (hallmark 8) but helps it evade and affect the immune cells, resulting in a tumor-promoting inflammation (hallmark 9). The subsequent metabolic changes alter cellular nutrient needs, while the growing tumor develops hypoxic areas, causing the cancer cells to induce vascularization (hallmark 5) which affects the influx of possible polymorphic bacteria (hallmark 12). Finally, tumor cells gain the ability to invade healthy tissue (hallmark 6), causing the tumor to spread throughout the body.

It should be strongly emphasized that this is only one possible scenario of tumor development. Not all hallmarks occur in every tumor cell, not all hallmarks occur simultaneously, the order of hallmark occurrence can vary, and each hallmark can push the cascade of tumor development as a whole. As a source of nutrients, blood vessel transport, microbes, different healthy cell types, and immune cells, the TME plays a major role in how fast the different tumor cells, and thus the tumor entity, have a chance to develop.

Based on this knowledge, a temporal treatment plan should be developed, and adapted specifically to each patient (Figure 9). This described treatment strategy is only one example for setting up a hypothetical rational strategy that acknowledges both the evolutionary dynamics of the tumor cells and the TME. Having neurosurgery/radiochemotherapy/alkylants as the only SoC approach, multiple combination treatment approaches can and should enter clinical research. The following described treatment strategy has been pioneered [26,128,129].

Globally, three different phases of combination therapies emerge, each including therapies focused against cancer cells, focused on the cancer-immune interaction and focused on the TME. The different phases ensure an effective response to an adaptable malignant glioma, a heterogeneous TME, and a changing systemic immune compartment. It allows for necessary timely adaptations, depending on current tumor status, and should thus be paired with continuous tumor monitoring.

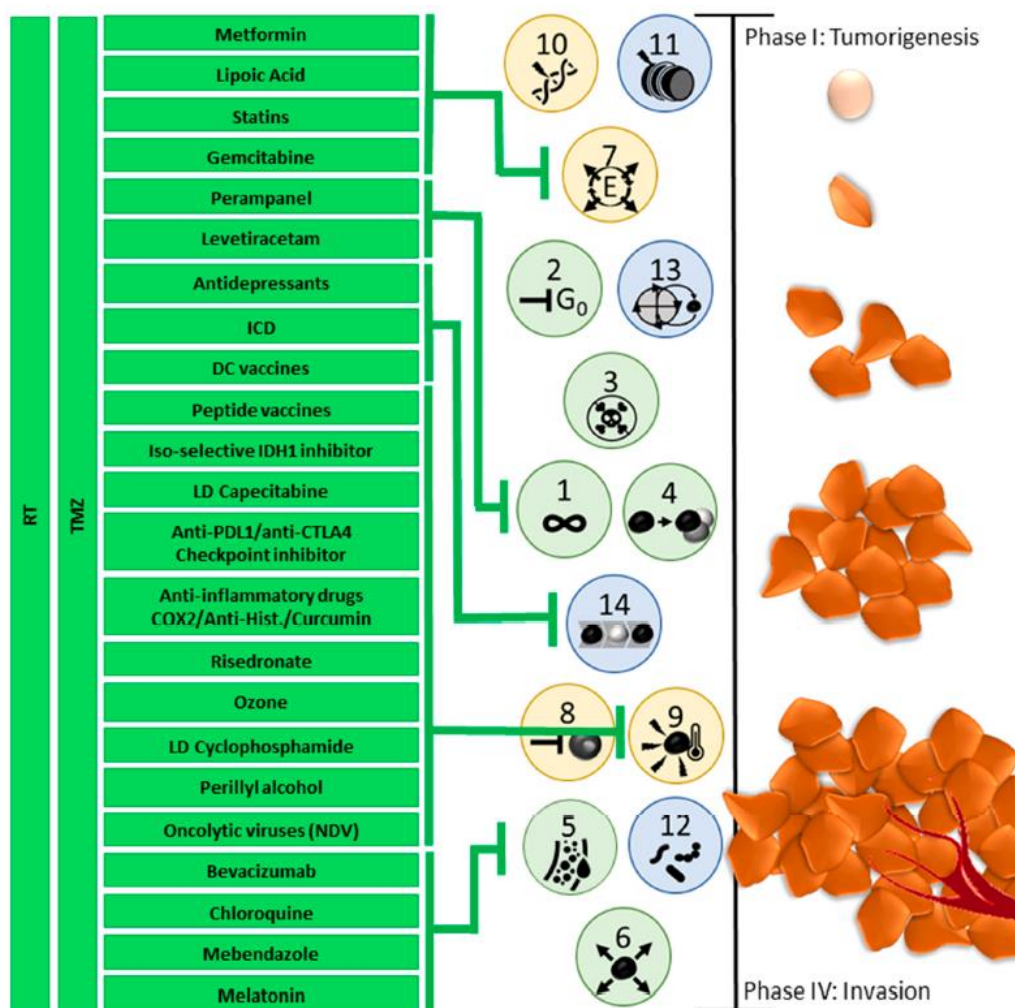


Figure 8. Example of temporal occurrence of individual tumor hallmarks during tumor development, from stage I, Tumorigenesis to stage IV, Invasion. Included is a temporal tumor treatment plan, to combat specific tumor hallmarks throughout malignancy development.

The first anticancer treatment phase (treatment phase I) aims to reduce tumor size, proliferation and infiltration via SoC treatment (surgery, RCT, and maintenance CTx) with 5 days of TMZ in 4-week cycles or Lomustin/TMZ in 6-week cycles. About 2 days after the maintenance chemotherapy course, immunogenic cell death (ICD) immunotherapy consisting of 5 daily sessions of modulated electrohyperthermia (mEHT) and bolus injections of oncolytic Newcastle Disease Virus (NDV) are given. ICD immunotherapy aims to kill cancer cells, including GSCs, via a biological and physics approach instead of only alkylating agents. As a result, the tumor burden reduces in size. The mEHT functions to prime the immune system in the TME and the inclusion of NDV ensures a cytotoxic immune response. Tumor Treating Fields (TTF), another type of physics therapy based

on electromagnetic waves, has already been added at this stage, resulting in improved prognosis [130]. Of note, part of the working mechanism of TTF has been suggested to be ICD-mediated tumor cell killing [131]. During this first anticancer phase, the use of repurposed drugs to affect the TME is proposed: anti-inflammatory drugs (Cox2 inhibition, NFkB inhibition, anti Histamin R1 drugs), a metabolic cocktail (Metformin, Lipoic acid, Atorvastatin), Mebendazole, drugs targeting the neuron-glioma axis, and melatonin. During treatment phase I, the administered RCT and CTx target immune cells besides the tumor cells, thereby weakening the immune system. In addition, the heterogeneous tumor is prone to mutate in response to treatment, with the formation of new subclones with a higher mutational burden. The treatment-induced combination of more aggressive tumor subclones with weakened immune surveillance is life-threatening. By adding ICD immunotherapy during the first anticancer treatment phase, the TME is prepared for a second phase of global treatment: immunization.

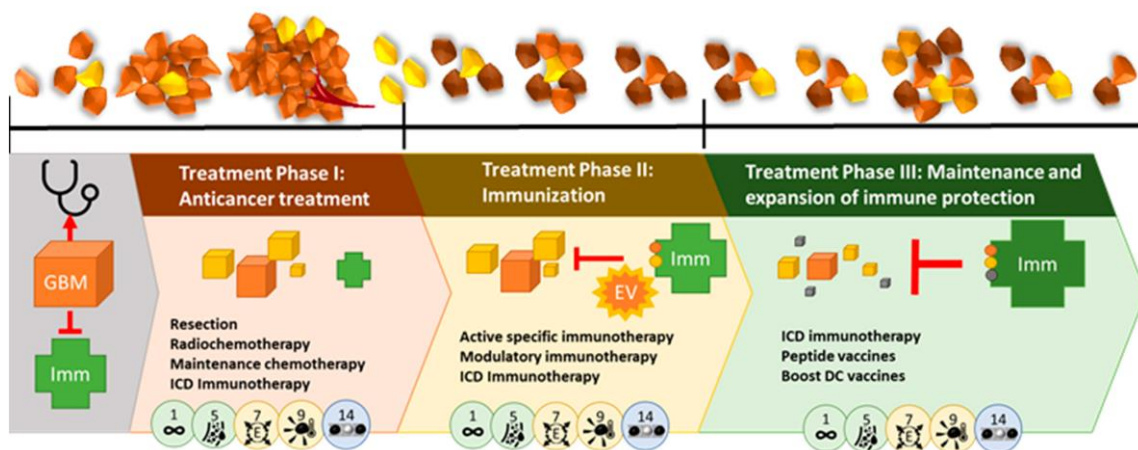


Figure 9. Overview of possible combination strategy trajectory, separated into three treatment phases that allow for adaptation and optimal patient-individualized treatment, based on tumor status and activity. GBM leads to symptoms (indicated as a stethoscope) and suppresses the immune system. Three subsequent treatment phases are proposed to combat the initial tumor and its escape mechanisms, by temporal targeting both, the tumor directly, and its TME. The gray–red–yellow–green background colors reflect treatment effectivity as increased tumor control, also shown as tumor dedifferentiation and size decrease at the top of the figure. Yellow tumor cells represent glioma stem cells, tumor cells in variations of brown (light to dark)

represent tumor cells of a different subtype, indicating here how a tumor of a different variation might grow after initial treatment of the main tumor subtype present, and how treatment might push further tumor differentiation.

Treatment phase II consists of active specific immunotherapy and modulatory immunotherapy to strengthen the immune system and train it against the heterogeneous residual tumor subclones and the GSC tumor cells. Patient-derived dendritic cells (DCs) are loaded with patient-specific tumor antigens. Either or both lysate from the original tumor tissue and ICD immunotherapy-induced serum-derived antigenic extracellular microvesicles and apoptotic bodies, also called large oncosomes [132], can be used to load the DCs. Both contain the spectrum of antigens that are present at time of vaccination, at the end of the first anticancer phase, during which radio- and chemotherapy might have altered tumor subclones and antigenicity. A cytokine cocktail (TNF- α , IL-1 β , IL-6) and the addition of NDV, which delivers viral antigen presentation and TLR stimulation, guarantee full DC maturation at time of DC vaccine release. After intradermal injection, the mature DCs reactivate endogenous T cells to target both the tumor itself and the NDV present inside the tumor after phase I. Concurrent modulatory immunotherapy consists of a patient-specific tailored combination of anti-inflammatory agents, checkpoint inhibitors (targeting, e.g., PD1-PDL1 interaction or CTLA4-CD80/CD86 interaction), and bisphosphonates (e.g., risedronate). The use of anti-PDL1 as a checkpoint inhibitor during this immunization phase might be favorable in the treatment of GBM, because of the particular targeting of the GAMs [83]. TME treatment with repurposed drugs is maintained during this phase. The scheduling of DC vaccination after maintenance CTx but not during CTx has been suggested to be beneficial [128,133].

Treatment phase III is aimed at the maintenance and expansion of immune protection, and is considered vital to keep the ever-changing tumor from flaring up. The tumor immune escape mechanism is a well-described phenomenon [26,134]. By combining ICD immunotherapy, peptide vaccines targeting more universally spread tumor antigens like WT1 [135] and survivin [136], but also specific antigens, like IDH1R132H [98] or H3 K27M [137], and booster DC vaccines, the immune system is repetitively trained against both current and newly emerging tumor antigens, targeting the cancer cells directly and indirectly from the outside and from within. Protection is updated and expanded, which is crucial to create long-term control over the malignant glioma. At this stage, the exhaustion of T cells might be overcome by using anti-PD1 and anti-CTLA4 checkpoint inhibitors. The question has risen whether recommended doses should be kept, or lower doses can be used [138,139]. Also, at this stage, TME treatment with repurposed drugs remains essential.

The potential effectiveness of this multiphase combined treatment strategy, which includes different modes of immunotherapy, has been reported. Overall survival data were compared to data of control arms of reported contemporary randomized clinical trials, outlining the current expected overall survival of these patients [26,140]. Though the clinical risk profiles of the reported real world patients were comparable or worse than the patient profiles in the randomized clinical trials, use of the proposed multiphase combined treatment strategy (Figure 9) resulted in a relevant increase in the 2 year overall survival in the adult population [26,140].

5. OPTIMIZING THERAPY REQUIRES CAREFUL CONSIDERATION AND CONSTANT MONITORING

The complexity of GBM treatment has been clearly established, and is reflected in its many possible treatment strategies. It is vital to consider that all aforementioned therapies do not function as a monotherapy, but rather need thoughtful combination or supplementation to effectively combat GBM. Combination therapies must be considered carefully, as some treatments have opposing effects or cancel each other out. To administer immunotherapy during chemo- or radiotherapy would defeat the purpose of the immunotherapy, as CTx and RCT both specifically target immune cells besides tumor cells [26,133]. Another example comprises the application of checkpoint inhibitors: in a randomized phase II trial and biomarker study, the PD1-checkpoint inhibitor pembrolizumab was administered alone (n = 30) or in combination with the anti-angiogenesis agent bevacizumab (n = 50) to patients with recurrent GBM [50]. Despite ample pre-clinical and clinical support that dual VEGF and immune checkpoint blockades might enhance anti-tumor immune responses, the authors reported no benefit of combination therapy. Their explanations for this lack of effect include that a potential complementary benefit is dependent on tumor context, and might not work for GBM specifically. A second reason given includes their use of a high bevacizumab dosage; lower dosing of antiangiogenics is related to vasculature normalization, while high doses are reported to augment hypoxia within the tumor, thus worsening immunosuppression. Antiangiogenics may also decrease the intratumoral penetration of therapeutic antibodies [50]. As anti-PD1 therapy is reported to trigger infiltration and activation of tumor-infiltrating lymphocytes in the TME, therapy timing might also play a major role [141].

Unfortunately, tumors have developed many ways of immunotherapy resistance. How the adaptive metabolism in the tumor and its TME affects immune cells through the expression of immunoregulatory factors was discussed in subchapters 2.4 and 2.5, but it was only recently discovered how metabolic restrictions, imposed by the TME, facilitate immunotherapy resistance and should thus be considered in both the development and application of immunotherapeutic strategies. It was found that specifically targeting metabolic deficiencies, such as hypoxia or the generation of suppressive metabolites, are promising in combination with GBM SoC [142].

The tumor-inherent mechanism of antigen escape is one of the main reasons immunotherapies such as chimeric antigen receptor (CAR)-T cells do not work on solid tumors, as CAR-T cells depend on recognition of surface antigens [143]. There exist seven different mechanisms of antigen escape: (1) pre-existing target-negative tumor clones, in which tumor cells with absent or dim antigen expression survive a first immunotherapy and then rise up to form a secondary tumor; (2) antigen gene mutations or alternative splicing, leading to loss or downregulation of antigens, or emergence of neoantigens against which the immunotherapy was not trained; (3) deficiencies in antigen processing, in which mutations in CD81 expression or CD19 regulation lead to an absence of CD19 in B cells required to recognize antigens; (4) antigen redistribution, in which antigens are transferred from the cell membrane surface to subcellular locations; (5) lineage switch, mainly described in leukemia patients where a lymphoid tumor transforms into a myeloid phenotype to escape treatment; (6) epitope masking, which occurs when tumor cells contaminate the harvested T cells and lead to CAR molecules which bind to CD19 on tumor cells and mask them; and (7) trogocytosis-mediated antigen loss, when cells acquire plasma from other cells, which can facilitate the transfer of antigens and lead to reduced antigen expression in tumor cells [143]. To overcome tumor immunotherapy resistance, it is deemed vital to understand the TME and

combine different immunotherapies to target the tumor in different ways and so combat its heterogeneity.

Another good example of the importance of well-considered combination therapy is THC treatment. Delta-9-tetrahydrocannabinol (THC) is a well-studied cannabinoid prescribed to alleviate the side effects of cancer treatment: loss of appetite, nausea, vomiting, pain, stress, and sleeplessness. THC is thought to have direct anticancer effects, promoting apoptosis in a Cannabinoid receptor-dependent manner, anti-angiogenesis, inhibiting proliferation and migration, and inducing cell cycle arrest [144]. Simultaneously, the use of THC might also have tumorigenic effects; the treatment of GBM U73-MG cell lines with THC resulted in increased cell proliferation [145]. In addition, use of THC is under suspicion of impeding immunotherapy. THC blocks JAK1 of the JAK-STAT pathway through cannabis receptor 2 (CNR2), which also blocks CD8+ T cell proliferation and anti-tumor immunity [146]. In an extensive retrospective study, cannabis use negatively impacted tumor response rate to nivolumab in 51 patients who used it in combination, as compared to 89 patients who solely used nivolumab [147], and was associated with a poorer clinical outcome [148]. Use of this ancillary drug during the first anticancer phase can be recommended, but in combination with immunotherapy is thus advised against [144,146–148].

There is an additional great potential in newly developed therapies, such as nucleic acid therapies. Nucleic acid therapies, such as mRNA, siRNA, miRNA, aptamers, circRNAs oligonucleotides, DNA, peptide nucleic acids, or cancer gene therapy, might be able to overcome hurdles that current immunotherapies cannot [149]. Initially explored for gene therapy, gene knockdown, and protein replacement, nucleic acid therapeutics have had promising results for hepatoblastoma, nephroblastoma, neuroblastoma, osteosarcoma, medulloblastoma, retinoblastoma, acute lymphoblastic leukemia and GBM [142]. Now, nucleic acids are being explored as immunotherapeutics for cancer. Nucleic acids are highly target-specific, small enough to cross the BBB, and they can directly affect the tumor or TME, or (re-)sensitize the malignancy to other combination therapies [150]. Patient-derived nucleic acids can easily be amplified in vitro, enabling therapy development from only small biopsy samples, and can be stored relatively well, making semi-off-the-shelf production possible. While DNA is more inexpensive and stable than RNA, it risks patient genome integration. On the other hand, nucleic acids need an efficient delivery method to avoid degradation by ubiquitous nucleases or triggering a local or systemic immune response. Nucleic acids can also cause off-target effects, have poor accumulation at tumor sites, and have a low blood stream circulation. To avoid this, delivery systems are under development [150].

This review is written in 2024/early 2025, and takes into account the currently available repurposed drugs that receive attention by the neuro-oncology community, for approaching five important hallmarks. It should be strongly acknowledged that other hallmarks are also focuses of novel treatment approaches. Particularly in the domain of malignant glioma, the hallmark of non-mutational epigenetic reprogramming is emerging as a targetable hallmark [151]. The gain of function proteins like mutant IDH or H3 can be approached. Vorasidenib, approved by the FDA for grade II astrocytoma [152], is already debated for its potential use in higher grade astrocytoma [153]. The efficacy of peptide vaccines against mutant IDH has been proven [98]. Targeting the gain-of-function mutants such as H3 K27M and H3 G34R remains challenging. Nevertheless, “epigenetic” therapies with HDAC inhibitors, DNA methylation inhibitors, and EZH2 inhibitors are still under development [154,155].

Though we offer a comprehensive overview over many factors that influence specific GBM mechanics, their interplay amongst themselves and the TME, there are still many other external influences which should be taken into account. In this review, we did not take external effects, such as family history, lifestyle habits or exposure to carcinogenic environmental factors into account. We excluded the microbiome from contemplation, though its importance is emerging [156,157]. On the topic of person-based effects, it must be noted that women possess enriched immunological signatures compared to men [158], which translates into a statistically significant 1 year survival advantage in female GBM patients. This effect was even more pronounced under the application of vaccine-based immunotherapy. Male GBM patients have a larger population of exhausted T cells [159]. To the best of our knowledge, the age at which the differences between sexes develop has not yet been researched. Beyond these factors that were not discussed, it must be taken into account that despite all we now know and learn about GBM and other brain malignancies using novel techniques, there is still much to discover.

One way to do this is by introducing more structured monitoring strategies. Current GBM and general cancer monitoring consist mainly of analytical snapshots. When a therapeutic plan is based only on a one-time assay, for instance on a pathological analysis after tumor resection, it ignores the many dynamic, swift changes in malignant glioma and its TME here outlined, often accompanied by treatment resistance. To actually enable proper adaptable treatment individualization, tumor and TME dynamics must be taken into account. Thus, we argue that tumor status analysis should not only commence with diagnostic procedures, but should be repeated regularly, in a similar way imaging and toxicity monitoring are. By monitoring the tumor as well as the patient and tumor immune state, cancer evolution can be detected and corrected earlier. Potential signs of treatment resistance can be caught and combatted with different therapeutic options, and a fast and accurate response is of vital importance. A treatment combination could be performed better when informed towards best effectivity. Moreover, continuous monitoring of GBM development would offer much more, and much more accurate, information to further improve GBM-treatment.

Liquid biopsy (LB) is a promising tool toward minimally invasive repeated monitoring of specific markers for disease and TME over the course of the disease. Distant from the original tumor, cell-free nucleic acids (cfDNA/RNA), extracellular vesicles (EVs) or circulating tumor cells (CTCs) can be harvested from blood, CSF, or urine. Several techniques to analyze samples derived from LB at time of diagnosis are commercially available [160–167]. Research programs under development or in clinical studies more often include LB, and are designed to evaluate the relevance of LB-monitoring in cancer evolution, therapy response and/or the onset of therapy resistance [168,169]. For over a century, GBM were classified only by their histological hallmarks: primary GBM development was considered to appear with no detectable precursors. Secondary tumors appeared to be so different from primary tumors, bearing mutually exclusive gene alterations, that they were considered two completely different tumor entities rather than a primary and secondary malignancy [170]. Molecular characterization through LB can support in GBM understanding: a current insight into actionable mutations or copy number aberrations of the tumor status can be obtained, and treatment can be adjusted accordingly [170]. However, the BBB poses a major obstacle. Rather than using non- or minimally invasive samples for LB, such as urine or blood, ctDNA must be harvested from CSF to obtain a high enough concentration for accurate analysis. By the time information can be obtained from blood samples, it is a clear indication of BBB breaking down [171], which unfortunately means treatment adjustments are occurring at the time of already

increased aggressiveness in the tumoral process. Beyond the low concentration, the absence of brain tumor markers poses another challenge. Commercial CTC systems rely on epithelial tumor markers for tumor cell identification, which are absent in brain tumors. Research currently includes H3 K27M [172,173], glial fibrillary acidic protein GFAP [165], or a mixture of SOX2, Tubulin beta-3, EGFR, A2B5, and c-MET [174], but with mixed success. More research is needed to improve LB of malignant glioma as a monitoring tool. However, new attempts are promising. One interesting approach is the measurement of GBM-derived EVs in the plasma as biomarkers for diagnosis, prognosis and monitoring [175]. Especially in the context of immunotherapy, longitudinal sampling of biofluids for multi-omics to dissect complex temporal changes in the GBM TME as a function of the immunotherapy is recommended [176].

Lastly, a multiphase combined treatment strategy is hypothesized and was tested against randomized clinical trials data. It is known that patients considered eligible for randomized clinical trials represent a thoroughly selected minority of patients in a realworld population [177]. Nonetheless, the application of the proposed multiphase combined treatment strategy with real world patients resulted in a relevant improvement of overall survival when compared to clinical trials [26,140]. It will be challenging to translate multiphase individualized combination treatment strategies into appropriate clinical trial strategies, so that the treatment approach becomes reproducible, for the benefit of all patients [19,177].

The implementation of individualized therapy does come with its own unique challenges. Though therapy options are improving, they often remain highly expensive due to their patient-specific nature. Often, individualized cell therapies cannot be prepared off-the-shelf, which means the need for a specific clean-room, specific equipment, trained staff, and GMP-certified processes within treatment facilities. Adaptation of personalized treatment would require a major rethink on treatment validation, as clinical trials usually require a standardized protocol in a standardized group of patients, both of which cannot apply [19]. These aspects complicate individualized therapy. However, it has also been shown that personalized treatment shortens the actual hospital or clinic stay, and vastly improves patient quality of life. Especially for GBM, the most frequent primary brain tumor in adults with the highest mean years of potential life lost amongst all human cancers [5], there is much to be gained.

Similarly, the use of repurposed drugs in GBM treatment should be considered carefully. It is often considered a fast-track in GBM treatment. As these drugs have already been applied to solve other medical problems, they often have been extensively tested for safety and pharmacokinetic properties. Repurposed drugs are often more readily available and relatively cheap compared to alternatives that may not yet be available [178,179]. However, the BBB can limit the ability of these drugs to reach the tumor site. Severe side effects can be expected when dealing with repurposed drugs. It must also be validated that the drugs can actually reach and affect the tumor [178,179].

6. CONCLUSIONS

In this review, we have discussed five tumor hallmarks. We outlined how they develop, what their molecular and cellular effects are, how they are affected by the TME and how they respond to treatment. It is clear that metabolic activity, nutrient availability, extracellular communication and hypoxia are cancer hallmarks which cause and contribute to the highly heterogeneous, spatio-

temporal fluctuating, fast-adapting character of GBM and its TME. An in-depth analysis of the many possible processes happening simultaneously in cancer intracellular maintenance and progression makes it clear that GBM functions as a complex regulated system rather than a simple cluster of independent fast-growing cells. The treatment responses and options listed indicate how multi-faceted treatment should be, in order to not only keep up with the tumor heterogeneity but to get ahead of it. To be able to effectively target the tumor as a whole, a thorough understanding of the cancer and its immediate surrounding tissue is required [140]. Several categories of repurposed drugs, and several modes of immunotherapy in combination with standard of care (SoC) are considered vital to treat not only the heterogeneous tumor but the specific GBM hallmarks. We believe it is vital to document and consider tumor location, metastases, primary or secondary, age, sex, MGMT promoter methylation status, mutations present or developing, and macrophage population, as these malignancy characteristics have a major influence on treatment strategy and response, and should be considered. However, initial documentation and consideration are not enough to treat an ever-changing tumor. We deem it critical to incorporate repeated monitoring. To properly combat the spatio-temporal aspects of GBM and its TME, repeated monitoring, for instance through liquid biopsy, of the tumor status and its development is necessary to ensure the treatment plan matches the actual tumorigenic aspects.

Further research will give more insight into other tumor hallmarks, and may lead to the development of accurate GBM subtype development models. Until that time, we depend on current patient information and treatment flexibility. Currently, tumor tissue from a first resection is often analyzed to determine post-surgery treatment. Primary in each treatment plan should be the extermination of GSCs, as these are highly treatment-resistant while offering GBM much of its heterogeneity. Antidepressants can be used against GSCs, and the TME must be carefully monitored and treated to avoid new GSCs developing. An individualized, multi-faceted, and multi-phased treatment plan should support the current SoC. With all the new developments in tumor understanding, tumor treatment cannot be left behind with only currently available SoC.

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ABBREVIATIONS

The following abbreviations are used in this manuscript:

TME	Tumor microenvironment
GSCs	Glioma stem-like cells
CNS	Central Nervous System
GBM	Glioblastoma
Tregs	T regulatory cells
NK	Natural Killer cells
GAMs	Glioma- or Tumor-associated microglia and macrophages, also known as TAMs
MDSC	Myeloid-derived suppressor cells
TAN	Tumor-associated neutrophils
BBB	Blood–brain barrier
SoC	Standard-of-Care
RCT	Radiochemotherapy
RT	Radiotherapy
CTx	Chemotherapy
Sc-seq	Single cell sequencing
miRNA	Micro-RNA
GTMEI	GBM-associated TIME immune cell infiltration
WHO	World Health Organization
Tcm	Central memory T cells
Tfh	T follicular helper cells
TMZ	Temozolomide
EMT	Epithelial-to-mesenchymal transition
VEGF	Vascular endothelial growth factor
EPCs	Endothelial progenitor cells
HIF	Hypoxia-inducible factor
CSF	Cerebrospinal fluid
LLMs	Lipid-laden macrophages
HMGB	high mobility growth factor
PDK	pyruvate dehydrogenase kinase
IL	Interleukin
ROS	Reactive oxygen species
OXPHOS	Oxidative phosphorylation
ACSS2	acetyl-CoA synthase
TGF	transforming growth factor
NO	Nitric oxide
FU	Fluorouracil
MMP	matrix metalloproteinases
CITE-seq	cellular indexing of transcriptomes and epitomes by sequencing
CyTOF	cytometry by time of flight
ER	endoplasmic reticulum
CPM	Cyclophosphamide
NDV	Newcastle Disease Virus
DMG	Diffuse midline gliomas
DIPG	diffuse intrinsic pontine glioma

pDHGG	diffuse pediatric-type high-grade glioma
H3 G34-mutant DHG	diffuse hemispheric glioma H3G34-mutant
H3 K27-altered DMG	diffuse midline glioma H3K27-altered
MRS	magnetic resonance spectroscopy
MRI	magnetic resonance imaging
DRD2	dopamine receptor D2
ClpP	mitochondrial caseinolytic protease P
α -KG	α -ketoglutaric acid
TNT	tunneling nanotubes
ICD	immunogenic cell death
mEHT	modulated electrohyperthermia
TTF	Tumor Treating Fields
DCs	Dendritic cells
LB	Liquid biopsy
EVs	extracellular vesicles
CTCs	circulating tumor cells
CAR-T cells	chimeric antigen receptor T cells

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CASE REPORT: EFFECTIVE MANAGEMENT OF RESIDUAL GLIOBLASTOMA WITH COMBINED MODALITY THERAPY

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Glioblastoma (GBM) is an aggressive primary brain tumor with a poor prognosis, often characterized by rapid progression and resistance to conventional therapies. This case report discusses the comprehensive management of a 60-year-old female diagnosed with residual GBM following initial surgical intervention. The treatment regimen included craniectomy, concurrent chemoradiotherapy (CCRT), adjuvant temozolomide, and weekly sessions of modulated electro-hyperthermia (mEHT, Oncothermia). Remarkably, the patient exhibited significant tumor shrinkage, improved neurological symptoms, and an extended survival period compared to typical outcomes. mEHT was utilized to enhance the efficacy of chemoradiotherapy and temozolomide by selectively targeting cancer cells and improving drug delivery. Integrating mEHT into the standard treatment protocol appears to have contributed to better therapeutic outcomes and improved quality of life for the patient. This case underscores the potential benefits of incorporating mEHT into multimodal treatment strategies for GBM, highlighting its role in enhancing the effects of conventional therapies. Future research and clinical trials are warranted to further explore the synergistic effects of mEHT with standard GBM treatments, aiming to establish more effective protocols and improve overall patient survival and quality of life. This report adds to the growing body of evidence supporting the use of innovative, integrative approaches in the management of aggressive brain tumors like GBM.

KEYWORDS

glioblastoma, oncothermia, modulated electro-hyperthermia, cancer management, brain tumor

INTRODUCTION

Glioblastoma (GBM) is the most common and aggressive primary malignant brain tumor in adults, accounting for approximately 47.7% of all primary malignant brain tumors (1). Despite advances in surgical techniques, radiotherapy, and chemotherapy, the prognosis for patients with GBM remains poor, with a median overall survival of 15 months following diagnosis (2). Standard treatment involves maximal safe resection followed by concurrent chemoradiotherapy (CCRT) with temozolomide (TMZ) and adjuvant temozolomide therapy. However, GBM's highly invasive nature, resistance to conventional treatments, and frequent recurrence pose significant challenges in achieving long-term control (3).

Modulated electro-hyperthermia (mEHT, Oncothermia) is an emerging treatment modality that uses low-intensity radiofrequency to selectively target cancer cells, enhancing the efficacy of chemotherapy and radiotherapy. By inducing cell death through apoptosis and mitotic catastrophe, mEHT enhances the damage caused by DNA-damaging agents (4, 5). mEHT operates by heating tumor tissues to approximately 40–42°C, which disrupts the cancer cells' ability to repair DNA damage, making them more vulnerable to concurrent therapies (6). Its use as an adjunct therapy in GBM has shown promising results in preclinical and clinical studies, with evidence suggesting that it may improve tumor control and prolong survival (7). mEHT has emerged as a promising adjunctive therapy in the treatment of various malignancies, including glioblastoma (GBM). Unlike conventional hyperthermia, which relies on the homogeneous heating of tumors, mEHT selectively targets malignant cells by exploiting the differences in electrical properties between cancerous and normal tissues. This selective heating leads to increased apoptosis, immune modulation, and improved tumor perfusion, enhancing the efficacy of chemotherapy and

radiotherapy. Clinical evidence supports the efficacy of mEHT in managing recurrent GBM, with studies demonstrating improved tumor control and survival outcomes.

The safety and efficacy of mEHT depend on optimized power delivery, ensuring that cancer cells are effectively targeted while sparing healthy brain tissue. Clinical studies have demonstrated that power levels up to 80 watts are safe for brain tumors, maintaining normal brain temperatures below 39.2°C while effectively increasing tumor perfusion and sensitizing cancer cells to radiation and chemotherapy (8, 9).

A retrospective multicenter controlled study by Fiorentini et al. (10) found that mEHT, when used in combination with integrative cancer therapies, contributed to prolonged survival and improved quality of life in patients with relapsed malignant glioblastoma and astrocytoma. While most evidence pertains to recurrent GBM, mEHT has shown potential as an adjunct therapy in newly diagnosed GBM, as it increases the sensitivity of cancer cells to standard treatments, potentially leading to better outcomes. Further investigation is warranted to confirm its efficacy in newly diagnosed cases (7). These findings support the potential of mEHT as an adjunct treatment in recurrent GBM, but there is limited data on its use in newly diagnosed cases.

In this report, we present a unique case of newly diagnosed GBM treated with a combination of standard CCRT and mEHT. This case highlights the potential for mEHT to enhance treatment outcomes in the initial management of GBM, rather than only in the recurrent setting. To our knowledge, this is the first case report documenting the use of mEHT in a newly diagnosed GBM patient, and it opens the door for further investigation into the efficacy of this treatment approach in earlier stages of disease.

CASE PRESENTATION

A 60-year-old female was diagnosed with GBM and underwent craniectomy and tumor removal in March 2023. However, after surgery, some tumor tissue remained. The patient received comprehensive treatment, including radiotherapy, chemotherapy, and mEHT. She has a medical history of dyslipidemia but no history of diabetes mellitus (DM), hypertension (HTN), hepatitis B virus (HBV), or hepatitis C virus (HCV). The patient occasionally consumes alcohol but does not smoke or chew betel nut. Additionally, there is no family history of cancer.

The patient is right-hand dominant. The symptoms began on January 22, 2023, with initial left palm numbness, lack of sensation in the fingers, a shooting-like sensation on the left side of the face, and neck pain. These symptoms lasted 5–10 minutes and recurred every 2–3 days.

Neurologically, the patient's right-hand muscle power was assessed as 5/5, and the left-hand muscle power was 4/5. Cranial nerves were intact, and the patient's Judgment, Orientation, Memory, Attention, and Calculation (JOMAC) were stable. There was no evidence of trunk ataxia, and all deep tendon reflexes (DTR) were normal.

The patient was diagnosed with glioblastoma after an MRI on March 7, 2023, and had surgery on March 13, 2023. Although the surgery reduced some of the tumor volume, a substantial portion of the tumor remains visible in the resection cavity, suggesting an incomplete resection. The

surrounding brain tissue exhibited significant edema, which was anticipated after the surgery and addressed in the subsequent treatment plan. Figure 1 illustrated that while the tumor resection reduced part of the tumor burden, a large residual tumor was still present, which was addressed through follow-up treatments, including chemoradiotherapy and hyperthermia. This highlights the necessity of continued multimodal treatment to control the remaining disease.

Before the surgery, the patient's Karnofsky Performance Scale (KPS) was around 80. Following the surgery, up until the MRI scan in June 2023, the patient experienced persistent swelling, and her KPS was around 50–60. However, from June to October 2023, the MRI showed a reduction in the swelling and continued shrinkage of the tumor, leading to an improvement in the KPS back to 80. By January 2024, MRI results indicated that the swelling had almost entirely resolved, and the patient's KPS had improved to 90. The primary remaining symptom at this time was numbness in the left palm, but the muscle power in the affected hand had returned to a score of 5.

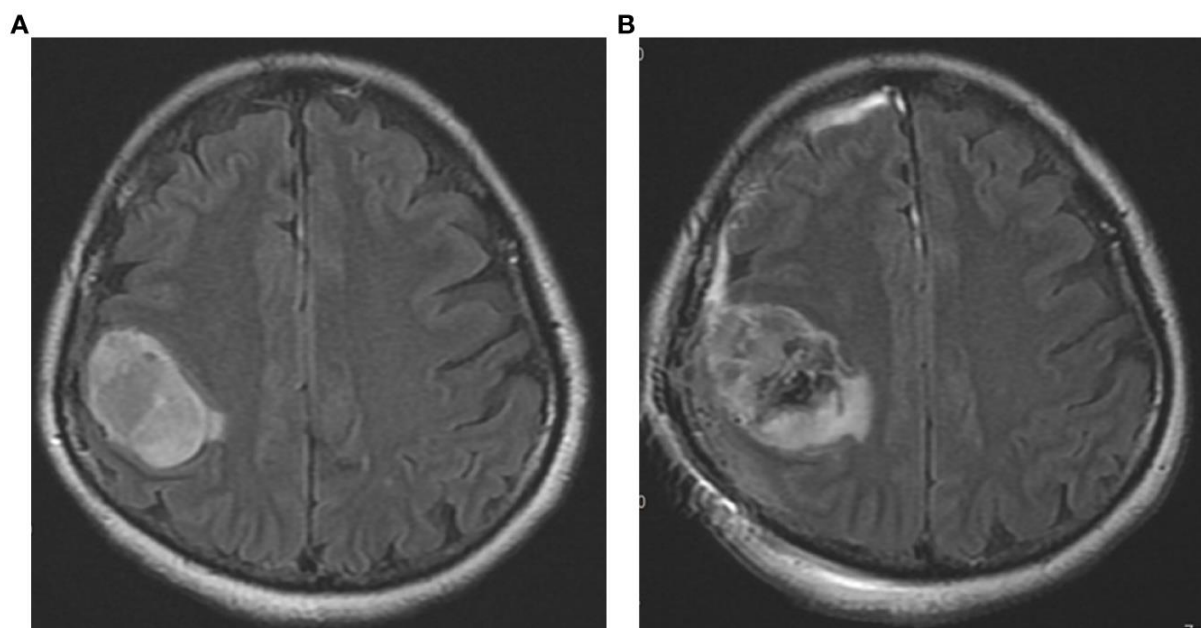


FIGURE 1

The pre-operative and post-operative MRI images of the patient's brain taken in T2/FLAIR sequences. (A) The preoperative image (dated 2023/3/7) shows a large tumor mass in the right parietal region. (B) The postoperative image (dated 2023/3/14) depicts the condition after surgical resection.

The patient had surgery on March 13, 2023, and was discharged on May 12, 2023. During her time in the hospital, the patient received radiotherapy and oral chemotherapy at the same time. She did not take steroids and did not have any seizure attacks. However, she was given preventive medications during and after her hospital stay, which included duloxetine 30 mg twice daily (BID), haloperidol 0.5 mg at bedtime (HS), quetiapine 25 mg at bedtime (HS), and levetiracetam 500 mg twice daily (BID).

Histologic Type and Location: The patient had a glioblastoma, IDH-wildtype (WHO Grade IV), located in the right parietal lobe.

Immunohistochemical Stains (IHC): The stains showed GFAP (+), IDH-1 (-), intact ATRX, and no significant overexpression of p53. However, data on MGMT methylation and NGS are not available.

Microscopic Description: The section showed widespread glial cell proliferation with increased cell density, abnormal cells, and significant mitosis. Necrosis and glomeruloid vascular proliferation were also observed.

Prognostic and Predictive Factors: Histologic grade: WHO Grade IV. The surgical margins could not be assessed, so they are considered positive.

RADIOTHERAPY

In Figure 2, the radiation field mapping for the patient's treatment plan is illustrated. Each panel represents a crosssectional image from a CT scan, showing the distribution of radiation dose across the targeted area. Figure 1 displays the planned dose for the patient's radiotherapy, with different colors representing different dose levels. Red marks the region receiving the full prescribed dose, while the surrounding color gradients indicate the gradual fall-off of the dose to nearby tissues. In Figure 1, the dose was modulated to minimize radiation exposure to critical structures surrounding the tumor while ensuring that the tumor receives the intended therapeutic dose. The total dose delivered was 6000 cGy across the region of interest, including the tumor and margins necessary to ensure adequate tumor control.

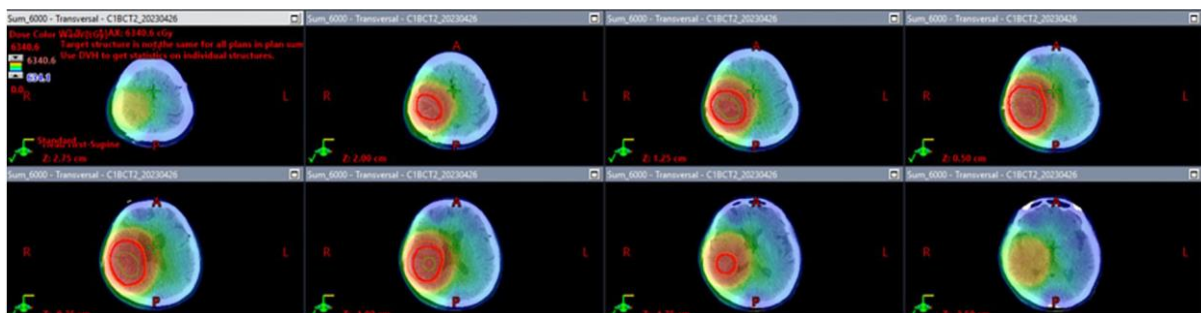


FIGURE 2

Radiation field mapping of glioblastoma treatment plan. The image displays radiation field mapping for the patient's radiotherapy treatment, showing how the radiation dose is distributed across the brain to target the tumor. The key details include the Gross Tumor Volume (GTV), Clinical Target Volume (CTV), and Planning Target Volume (PTV). The total prescribed dose is 6000 cGy, with color gradients indicating how the dose tapers off around the tumor, ensuring effective targeting of tumor cells while minimizing exposure to healthy tissues.

The patient began radiotherapy on March 30, 2023, and completed it on May 11, 2023. She received a total of 30 fractions with a cumulative dose of 6000 cGy. The radiotherapy was carried out in two phases. In the first phase, a 2-centimeter margin was added to the clinical target volume (CTV) to accommodate peripheral edema, and a dose of 4600 cGy was administered. In the second phase, the treatment area was reduced to specifically target the residual tumor area without edema, and an additional boost of 1400 cGy was given, resulting in a total dose of 6000 cGy. No steroid was used during radiotherapy.

The post-radiotherapy MRI image, taken on June 6th, 2023 (Figures 3A, 4A), shows the tumor following concurrent chemoradiotherapy (CCRT) treatment. At this stage, the T2/ FLAIR sequence reveals a decrease in tumor size with surrounding edema still present. The therapeutic response to radiotherapy is evident, as there is a reduction in the tumor's intensity compared to the pre-treatment scans. However, the presence of residual tumor and edema suggests the need for continued monitoring and additional treatments.

CHEMOTHERAPY

After undergoing radiotherapy, the patient was given a daily dose of 120 mg of Temozolomide. The patient's body surface area (BSA) was calculated based on her height (161 cm) and weight (64 kg), resulting in a BSA of 1.62 m². According to standard dosing guidelines, she should have received a dose of 75 mg/m² of Temozolomide, which equals 121.5 mg daily. However, since Temozolomide is only available in 100 mg and 20 mg formulations, she was administered 120 mg daily during radiotherapy as part of the concurrent chemoradiotherapy (CCRT) protocol.

Following the completion of radiotherapy, the patient started an adjuvant Temozolomide regimen, taking 340 mg daily for 5 days every 28 days. A 200 mg/m² dose was given, equal to 330 mg daily. However, due to the available dosage forms (100 mg and 20 mg tablets), the daily dose was rounded to 340 mg. This post-radiation chemotherapy treatment began on July 6, 2023.

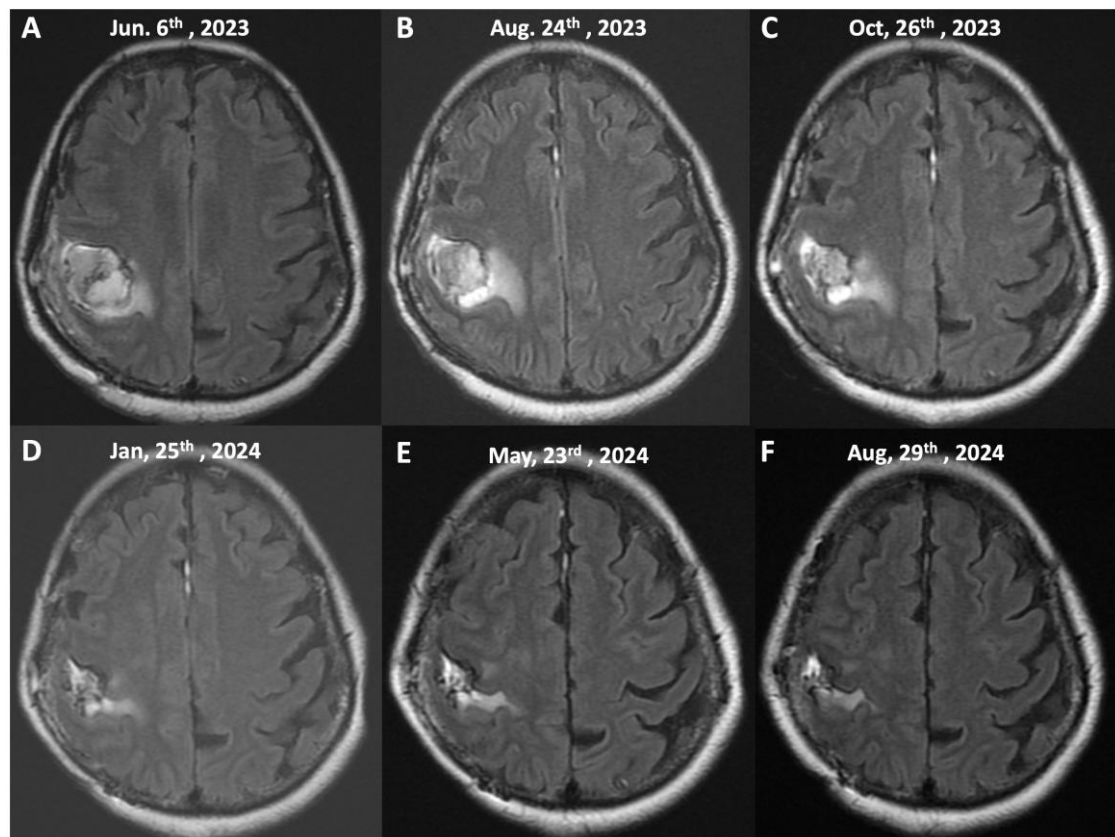


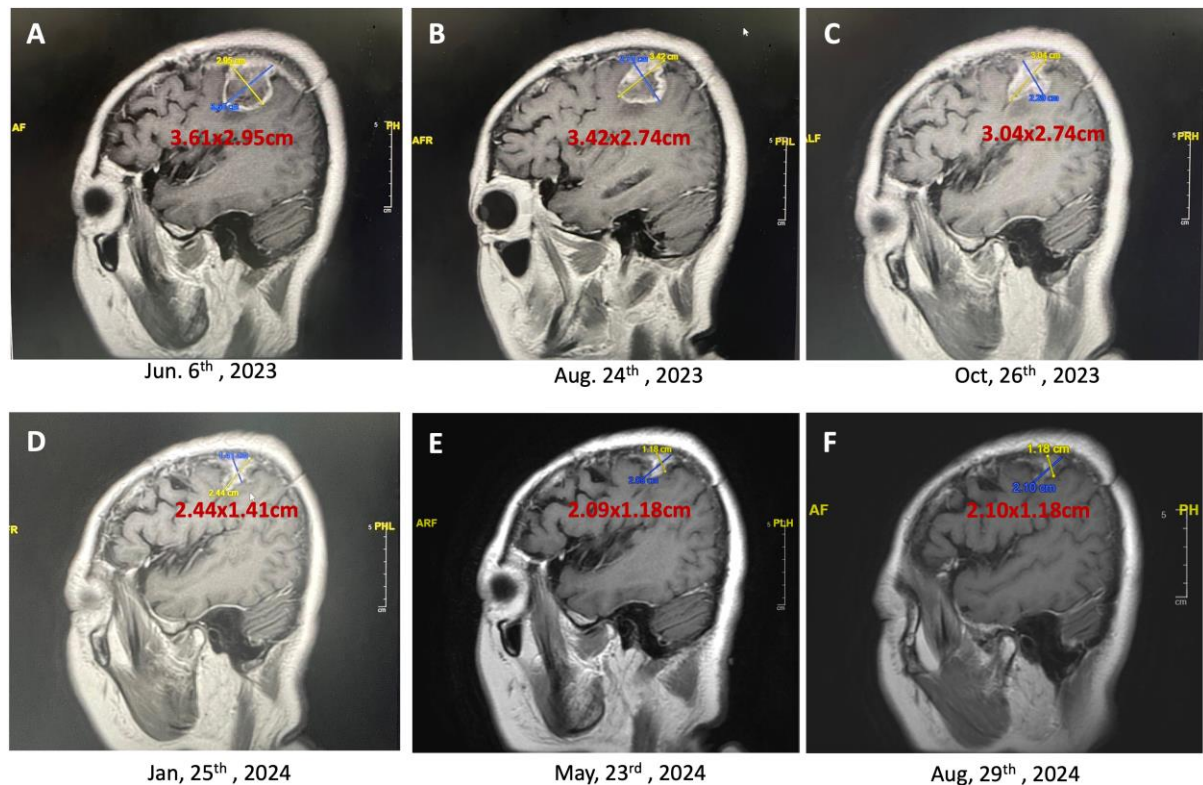
FIGURE 3

Serial MRI imaging of tumor progression and response to treatment (T2/FLAIR Sequences, Axial View). This figure illustrates the sequential MRI images of the patient's brain taken over a 15-month period, demonstrating the tumor's response to treatment, including chemoradiotherapy

(CCRT) and continuous mEHT therapy. The images show axial T2/FLAIR sequences at different stages of the patient's treatment course: (A) (June 6th, 2023), (B) (August 24th, 2023), (C) (October 26th, 2023), (D) (January 25th, 2024), (E) (May 23rd, 2024), (F) (August 29th, 2024).

All the dates the patient received adjuvant Temozolomide were listed: 2023/6/7–6/11, 7/6–7/10, 8/1–8/5, 8/31–9/4, 9/28–10/2, 10/26–10/30, 11/23–11/27, 12/21–12/25, 2024/1/18–1/22, 2/15–2/19, 3/14–3/18, 4/11–4/15, 5/9–5/13, 6/6–6/10, 7/4–7/8, 8/1–8/5, and 8/29–9/2.

The patient continues this regimen as her disease remains stable without progression.



FIGURES 4

Persistent reduction in brain tumor size over time (T1 contrast-enhanced MRI, sagittal view). This series of MRI images demonstrated the continuing reduction in the size of a brain tumor in a 60-year-old female patient undergoing a combination of craniectomy, chemoradiotherapy (CCRT), adjuvant temozolomide, and weekly mEHT sessions. (A), The initial MRI scan shows a tumor size of 3.61 x 2.95 cm (June 6, 2023); (B), Following two months of treatment, the tumor size reduced to 3.42 x 2.74 cm (August 24, 2023); (C), Further reduction in tumor size to 3.04 x 2.74 cm is observed (October 26, 2023); (D), Continued treatment resulted in a significant reduction in tumor size to 2.44 x 1.41 cm (January 25, 2024); (E), The latest MRI scan shows the tumor has shrunk to 2.09 x 1.18 cm (May 23, 2024), demonstrating the effectiveness of the multimodal treatment approach in reducing tumor size and improving patient prognosis. (F), The final MRI shows the tumor size stabilizing at 2.10 x 1.18 cm (August 29, 2024) with minimal contrast enhancement, indicating the effectiveness of the ongoing mEHT therapy in maintaining disease stability.

MEHT

The patient underwent mEHT (Oncothermia) as part of her treatment once a week consistently without any breaks. Weekly mEHT sessions began on May 24, 2023, and as of September 12, 2024, 67 mEHT sessions had been completed. Each session lasted for 60 minutes. The mEHT machine used for the treatments was the EHY-2000 model, operating at a frequency of 13.56 MHz. The power output was kept mainly at 80 watts to target cancer cells while preserving healthy tissues.

The mEHT treatments were specifically administered to the site of the brain. This approach utilizes controlled electromagnetic fields to selectively increase the temperature of cancer cells, causing stress on their cell membranes and leading to cell death. This selective heating enhances the effectiveness of concurrent treatments, such as immunotherapy and chemotherapy, by making the cancer cells more susceptible to these therapies. The treatment was welltolerated, and no significant adverse events or complications were reported during therapy. The peripheral edema and tumor both gradually reduced in size, so no steroids or Avastin (bevacizumab) were administered. Figures 3, 4 showed the effects of mEHT on the patient's tumor size over time.

TUMOR STATUS

Initial imaging post-CCRT and mEHT demonstrated a residual tumor measuring 3.61 x 2.95 cm. Subsequent imaging at thirteen months post-surgery revealed a significant reduction in tumor size to 2.10 x 1.18 cm. The tumor continued to shrink without associated edema.

SYMPTOMS AND QUALITY OF LIFE

The patient first experienced symptoms on January 22, 2023, which included numbness in the left palm, a lack of sensation in the fingers, a shooting-like sensation on the left side of the face (hemiface), and neck pain on the left side. These symptoms lasted 5-10 minutes before disappearing but would return every 2-3 days. Upon examination, hand muscle strength was rated at five on the right and four on the left. The patient's cranial nerves were intact, their judgment and memory (JOMAC) were stable, no signs of trunk ataxia were present, and deep tendon reflexes (DTR) were normal.

An MRI scan confirmed the diagnosis of the tumor on March 7, 2023, and surgery was performed on March 13, 2023. Before surgery, the patient's Karnofsky Performance Scale (KPS) score was approximately 80. Following the surgery, the MRI scans up until June 2023 still showed some edema; during this time, the KPS score dropped to 50-60. From June to October 2023, the MRI scans indicated a reduction in the edematous area, and the tumor continued to shrink, leading to an improvement in the patient's KPS score to 80. By January 2024, the MRI showed that the edema had nearly completely resolved, and the patient's KPS score returned to 90. The primary residual symptom was numbness in the left palm, but muscle strength had fully recovered to a score of 5.

A timeline of the patient's symptoms and changes:

- 2023/1/22:
 - Initial onset of symptoms, including numbness in the left palm, lack of sensation in the fingers, shooting pain on the left hemiface, and left neck pain.
 - Symptoms persisted for 5–10 minutes and returned every 2–3 days.
 - Hand muscle strength: right hand = 5/5, left hand = 4/5.
 - Cranial nerves were intact, JOMAC stable, no trunk ataxia, DTRs were normal.
- 2023/3/7:
 - MRI confirmed the diagnosis of the tumor.
- 2023/3/13:
 - Surgery performed.
 - Pre-surgery Karnofsky Performance Scale (KPS) score was approximately 80.
- 2023/3 – 2023/6:
 - Post-surgery, MRI scans showed persistent edema.
 - KPS score dropped to approximately 50–60 during this period.
- 2023/6 – 2023/10:
 - MRI scans showed a reduction in the edematous area and continued tumor shrinkage.
 - KPS improved to 80 during this time.
- 2024/1:
 - MRI showed that the edema had almost completely resolved.
 - KPS score returned to 90.
 - Residual symptoms included left palm numbness, but hand muscle strength had fully recovered to a score of 5/5.

SURVIVAL

The patient exhibited remarkable survival and disease control, with a stable disease state lasting 16 months following initial resection. While a 14-month survival post-resection may not be extraordinary by current standards, the key observation in this case is the extended progression-free interval (PFI) of 16 months. This extended PFI was achieved without additional aggressive interventions such as salvage surgery, re-irradiation, second-line bevacizumab, or tumor treating fields (TTF). The patient continued to receive standard concurrent chemoradiotherapy with temozolomide and underwent weekly mEHT sessions as the only supplementary therapy.

During this time, the patient's KPS remained high, and she demonstrated no significant disease progression on follow-up imaging. MRI scans indicated stable disease and a gradual reduction in tumor size and surrounding edema (as shown in Figures 3, 4). Importantly, the patient's PFI extended beyond expectations without requiring further invasive or aggressive treatments, highlighting the potential impact of continuous mEHT in maintaining disease control.

This outcome suggests that mEHT may have contributed to delaying disease progression when combined with standard GBM treatment protocols. Although this is a single case, the extended survival without progression underscores the need for further investigation into the role of mEHT in glioblastoma management, significantly as a complementary therapy to enhance standard treatment outcomes.

DISCUSSION

This case highlights the feasibility and potential of using modulated electro-hyperthermia (mEHT) as an adjunct therapy in the management of newly diagnosed glioblastoma (GBM). While most of the evidence regarding the use of mEHT has been in the recurrent setting, the addition of mEHT in this case for a newly diagnosed patient provides preliminary insight into its safety and potential application earlier in the treatment continuum. However, it is important to emphasize that this case does not establish definitive efficacy but rather highlights the feasibility of integrating mEHT with standard treatments such as concurrent chemoradiotherapy (CCRT) and temozolomide (TMZ) for newly diagnosed GBM patients.

In this case, combining mEHT with CCRT and adjuvant temozolomide correlated with a reduction in tumor size and stabilization of the disease. However, it should be noted that this observation is speculative, and it is difficult to conclusively attribute this outcome solely to the addition of mEHT. GBM is a highly aggressive tumor with a poor prognosis, and reductions in tumor size may be influenced by multiple factors, including the effects of chemoradiotherapy, the patient's MGMT methylation status, and other biological variables. Therefore, while mEHT may have played a role in this case, further controlled studies are necessary to understand the extent of its contribution to tumor reduction and disease control.

The patient in this case demonstrated IDH-wildtype, which is associated with a worse prognosis. Unfortunately, MGMT methylation status was not available, which leaves an incomplete assessment of the patient's molecular profile. Patients with MGMT-methylated tumors typically respond better to temozolomide, and the absence of this data limits our understanding of the full therapeutic potential in this case. Therefore, it is difficult to determine whether the observed progression-free interval (PFI) was primarily due to mEHT or the effects of standard therapies, such as CCRT and adjuvant temozolomide.

Additionally, the patient experienced notable improvements in symptoms such as headache, speech fluency, and motor function, which improved her overall quality of life. However, it is important to acknowledge that the improvement in symptoms may not be entirely attributable to mEHT, as they could be related to the effects of radiotherapy, chemotherapy, and other aspects of the overall treatment plan. Therefore, while mEHT may contribute to improving patient outcomes, we cannot definitively claim that it was the primary factor responsible for symptom relief in this case. In terms of survival, the patient has achieved a stable disease state 14 months post-resection, which is a positive outcome in the context of GBM. However, given the typical aggressive nature of the disease, a 14-month survival period should not be considered exceptional. The available literature on mEHT in the recurrent setting has suggested that it may improve progression-free and overall survival, but these findings are largely based on recurrent cases and retrospective studies. As this case was treated in the newly diagnosed setting, it provides an

opportunity to investigate whether the early application of mEHT could yield similar or improved results in prospective studies.

The sagittal MRI scans clearly show a gradual decrease in the tumor dimensions, but this could partly be attributed to the retraction of the surgical cavity following resection, rather than solely due to mEHT treatment. The immediate post-operative MRI revealed a substantial resection cavity, and as the tissue healed, the cavity size may have contributed to the observed reduction in the MRI images. Therefore, while the tumor appeared smaller over time, we cannot definitively attribute this effect to oncothermia. More robust imaging follow-up, including tissue-based analyses, would be needed to clarify whether mEHT directly contributed to the decrease in tumor size.

Hyperthermia offers several benefits for treating brain tumors. First, it enhances the effectiveness of conventional treatments like chemotherapy and radiotherapy by increasing the permeability of the blood-brain barrier, allowing for better drug delivery to the tumor site. Second, hyperthermia induces the expression of heat shock proteins, which can stimulate the immune system to recognize and attack tumor cells more effectively. Third, it directly causes thermal damage to cancer cells, leading to their apoptosis or necrosis, while sparing surrounding healthy tissue due to differential heat sensitivity. Fourth, hyperthermia can disrupt the repair mechanisms of cancer cells, making them more susceptible to radiation- and chemotherapy-induced damage. Finally, when combined with immunotherapy, hyperthermia can enhance the immune response against the tumor, improving overall treatment outcomes (11, 12). Further research and clinical trials are warranted to validate these findings and optimize treatment protocols (11, 13).

Current literature on the use of mEHT in GBM primarily focuses on patients with recurrent disease, where its efficacy has been suggested in retrospective studies as a complementary therapy to standard care (10, 14). However, there is limited evidence for its use in newly diagnosed patients. This case demonstrates the feasibility of integrating mEHT early in the treatment course, alongside chemoradiotherapy and temozolomide, without adding significant toxicity. Nevertheless, without tissue analysis posttreatment or additional biomarkers of response, it is impossible to definitively conclude the effectiveness of mEHT in prolonging survival or improving outcomes. This case underscores the importance of conducting clinical trials to explore mEHT's role in enhancing therapeutic efficacy in the newly diagnosed setting.

One of the key aspects of mEHT treatment is optimizing power delivery to ensure therapeutic efficacy while maintaining the safety of normal brain tissue. In this study, we maintained power levels below 80 watts, a setting that has been shown in previous clinical trials to be safe and effective for glioblastoma treatment. Clinical evidence suggests that power levels at or below 80 watts selectively target tumor cells while keeping normal brain temperatures below the threshold of 39.2°C, thereby minimizing the risk of thermal damage to healthy tissue (8, 9). Additionally, maintaining this power range optimizes tumor perfusion, enhances oxygenation, and increases radiosensitivity by inhibiting DNA repair processes. This approach is supported by studies demonstrating that lower but sustained hyperthermia temperatures (40–42°C) effectively sensitize tumor cells to radiation and chemotherapy without causing significant neurotoxicity (15).

In conclusion, while this case demonstrates the safety and feasibility of combining mEHT with standard GBM treatments, it does not provide conclusive evidence of its efficacy in prolonging survival or improving disease control. More extensive clinical trials with appropriate controls,

biomarkers, and long-term follow-up are necessary to fully understand the potential benefits of mEHT in newly diagnosed GBM patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the studies involving humans because the patient provided written informed consent to participate in this study. The studies were conducted in accordance with the local legislation and institutional requirements. The participant provided her written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

H-JC: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Writing – review & editing. C-HK: Investigation, Methodology, Project administration, Resources, Writing – original draft. Y-SW: Software, Supervision, Validation, Visualization, Writing – original draft. Y-CK: Conceptualization, Formal Analysis, Methodology, Visualization, Writing – review & editing.

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Conflict of interest

Author Y-SW was employed by the company Uni-Pharma Co-Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHEMORADIOTHERAPY COMBINED WITH MODULATED ELECTRO-HYPERTHERMIA (mEHT) CONVERTS INITIALLY INOPERABLE ESOPHAGEAL CANCER TO OPERABLE DISEASE

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KEYWORDS

advanced cancer management | chemoradiotherapy | esophageal cancer | modulated electro-hyperthermia | tumor downstaging

ABSTRACT

This case report demonstrated the effectiveness of combining chemoradiotherapy (CCRT) with modulated electro-hyperthermia (mEHT) in converting initially inoperable esophageal cancer into an operable condition. A 60-year-old male with stage IVB lower third esophageal squamous cell carcinoma (cT3N2M1) experienced significant tumor shrinkage (ypT2N0M0) and a notable metabolic response, which enabled successful surgical removal. This treatment approach highlights the potential of mEHT as a valuable addition to the comprehensive management of esophageal cancer.

1. INTRODUCTION

Esophageal cancer remains a significant global health challenge due to its high mortality rate and complex management [1]. Despite advancements in medical research and treatment methods, the prognosis for patients with esophageal cancer, particularly those in advanced stages, remains grim [2]. Conventional treatments, such as surgery, chemoradiotherapy, and targeted therapies, often achieve limited success, primarily because of the disease's aggressive nature and its propensity for early metastasis [3]. This highlights the urgent need for more effective treatment strategies to improve patient outcomes.

Modulated electro-hyperthermia (mEHT) is an innovative adjunct therapy that has demonstrated promise in enhancing the efficacy of traditional cancer treatments [4]. By delivering localized heat through electromagnetic waves, mEHT induces cancer cell death via apoptosis and mitotic catastrophe while amplifying the repair of DNA damage caused by chemotherapy and radiotherapy [5]. The therapeutic potential of mEHT lies in its ability to selectively target malignant cells, minimizing harm to surrounding healthy tissues and improving overall treatment effectiveness.

Previous studies have explored the application of mEHT in various cancers, including esophageal cancer. Clinical trials have indicated that mEHT, when combined with chemoradiotherapy, can enhance tumor response rates and improve survival outcomes [4]. However, integrating mEHT into standard treatment protocols for esophageal cancer remains an area of active investigation, with further clinical evidence required to confirm its efficacy and safety.

This case report describes a 60-year-old male diagnosed with initially inoperable stage IVB esophageal squamous cell carcinoma. Through a combination of concurrent chemoradiotherapy (CCRT) and mEHT, the patient achieved a robust pathological response, enabling successful surgical resection. This case underscores the potential of mEHT as an effective adjunct therapy for advanced esophageal cancer, offering renewed hope for patients with limited treatment options.

2. CASE REPORT

A 60-year-old male presented with a three-month history of a left neck mass. On examination at the ear, nose and throat (ENT) outpatient department, the mass was firm, movable, non-tender, and measured 2 × 2 cm, located at level V of the left neck. Ultrasound-guided fine-needle aspiration of the mass confirmed metastatic carcinoma. Further evaluation with neck/chest CT revealed the following findings: a long segmental wall-thickening mass in the lower third of the esophagus, measuring over 7.5 cm in length and 2 cm in thickness, suspicious for esophageal carcinoma; three to six regional metastatic lymph nodes in the left gastric artery and paraesophageal spaces; and non-regional metastatic lymphadenopathy in the left neck levels IV and V.

Endoscopic ultrasound with biopsy further confirmed advanced esophageal squamous cell carcinoma involving the middle and lower esophagus, with extension into the esophagogastric junction (EGJ). 18F-fluorodeoxyglucose Positron Emission Tomography/Computed Tomography (PET/CT) revealed a viable tumor in the lower esophagus, regional nodal metastases in the paraesophageal and celiac regions, and distant nodal metastases in the left supraclavicular fossa (SCF) and left neck level V. The SUV max of the largest metastatic node was 7.3. Based on these findings, the patient was diagnosed with lower esophageal squamous cell carcinoma, cT3N2M1, stage IVB.

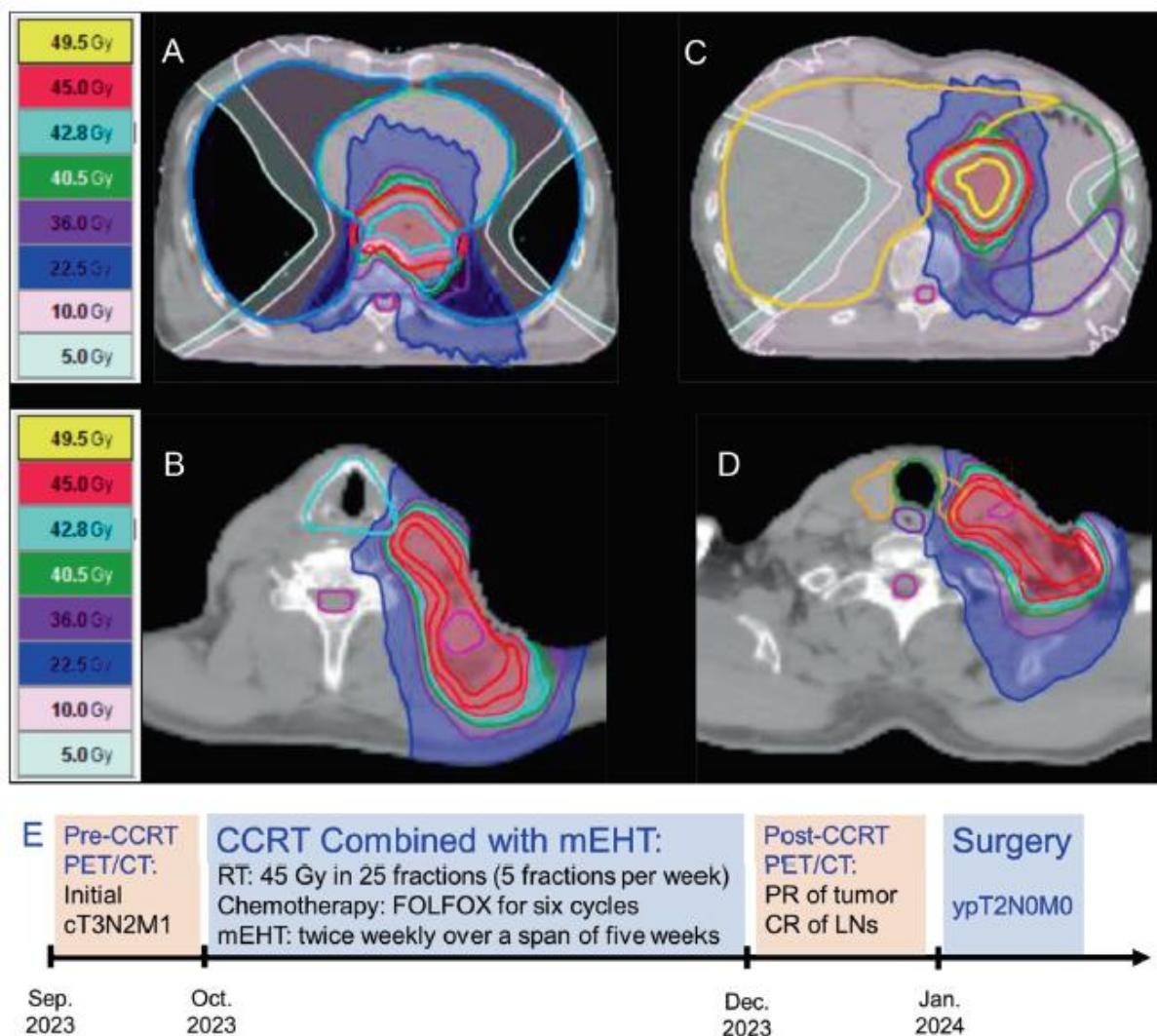


FIGURE 1 | Radiation dose distribution from the radiotherapy treatment plan and an overview of the clinical course for a 60-year-old male diagnosed with lower esophageal squamous cell carcinoma, cT3N2M1, stage IVB. The planning target volume (PTV) of 45 Gy (red area) is focused on the primary esophageal tumor in the lower third of the esophagus (A), the left neck level V nodal metastasis (B), the celiac nodal metastasis (C), and the left supraclavicular fossa nodal metastasis (D). This distribution targets both the primary tumor and regional as well as distant metastatic lymph nodes identified on PET/CT and CT imaging. (E) an overview of the patient's clinical course. CCRT: Concurrent chemoradiotherapy. mEHT: Modulated electro-hyperthermia. PET/CT: Positron Emission Tomography/Computed Tomography. PR: Partial response. CR: Complete response.

Given the extent of the disease and distant nodal metastases, surgery was not recommended by the thoracic surgery team. Consequently, the treatment plan included concurrent chemoradiotherapy (CCRT). Over a two-month period, the patient received six cycles of FOLFOX chemotherapy and radiotherapy, delivering a total dose of 4500 cGy in 25 fractions, targeting the primary esophageal tumor and the metastatic nodes in the paraesophageal, celiac, and left SCF regions. The goal of mEHT in oncology is to elevate the tumor temperature within the 39°C–42.5°C range, thereby enhancing its susceptibility to oncologic therapies [4]. The mEHT procedure for this patient was conducted using the EHY2030 device, administered twice weekly over a span of 5 weeks. Each session lasted 60 min, following a gradual escalation protocol to optimize treatment efficacy. The patient was positioned supine, with a 30 cm circular electrode applicator placed on the chest wall, precisely targeting the esophageal tumor. The stepwise power adjustment began at 80 W for the initial 10 min, progressively increasing to 100 W for the subsequent 10 min, and further escalating to 180 W for another 10 min. During the final 30 min of treatment, the power output was modulated based on the patient's tolerance and subjective discomfort, ensuring safety while maintaining therapeutic efficacy, with a maximum threshold of 240 W. Figure 1 illustrates the radiation dose distribution, with a planning target volume (PTV) of 45 Gy covering the primary tumor and metastatic lymph nodes, along with an overview of the patient's clinical course.

A follow-up PET/CT scan, performed approximately 6 weeks after completing CCRT and mEHT, demonstrated a partial metabolic response (PMR) in the primary esophageal tumor and a complete metabolic response (CMR) in the nodal metastases. The initial PET/CT had revealed a 54-mm mass in the anterior mediastinum with an SUVmax of 9.4. Following treatment, the SUVmax of the primary tumor decreased to 5.4, indicating PMR <https://www.youtube.com>(Figure 2A,B). Significant metabolic reductions were observed in the nodal metastases, including the left SCF node (SUVmax reduced from 4.8 to 1.3; Figure 2C,D), the celiac node (SUVmax reduced from 3.5 to 1.3; Figure 2E,F), and the left neck level V nodes (SUVmax reduced from 7.3 to 0.6; Figure 2G,H).

Two months after completing CCRT and mEHT, the patient underwent video-assisted thoracoscopic surgery (VATS) esophagectomy with gastric tube reconstruction, VATS lymph node dissection, and feeding jejunostomy. Postoperative pathology revealed ypT2N0M0, indicating no residual nodal involvement and significant tumor downstaging. This outcome represented a successful conversion of an initially inoperable stage IVB disease to an operable condition.

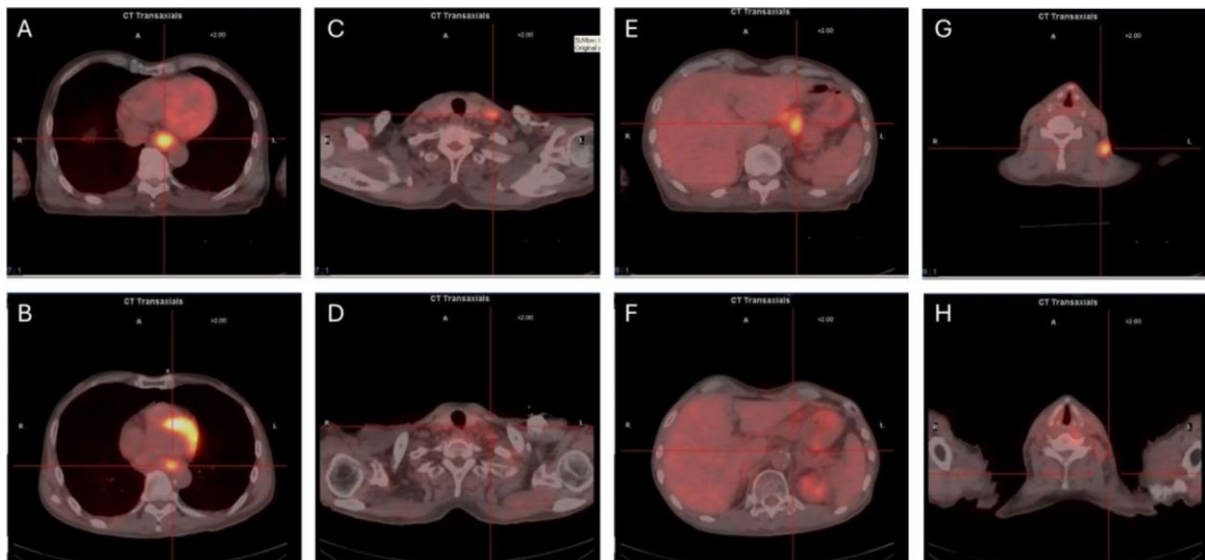


FIGURE 2 | PET-CT images illustrating the metabolic response of the primary esophageal tumor and nodal metastases before and after treatment with chemoradiotherapy and modulated electro-hyperthermia. (A, B) Primary esophageal tumor before treatment (A, SUVmax 9.4) and after treatment, showing a partial metabolic response (B, SUVmax 5.4). (C, D) Left supraclavicular fossa nodal metastasis before treatment (C, SUVmax 4.8) and after treatment, showing a complete metabolic response (D, SUVmax 1.3). (E, F) Celiac nodal metastasis before treatment (E, SUVmax 3.5) and after treatment, showing a complete metabolic response (F, SUVmax 1.3). (G, H) Left neck level V nodal metastasis before treatment (G, SUVmax 7.3) and after treatment, showing a complete metabolic response (H, SUVmax 0.6).

3. DISCUSSION

Esophageal cancer classified as cT3N2M1, stage IVB, is widely regarded as unsuitable for surgical intervention at diagnosis due to its advanced stage and extensive lymph node metastasis, with outcomes remaining poor even after CCRT [2, 3]. This case highlights the effectiveness of combining mEHT with CCRT in treating advanced esophageal cancer. The integration of mEHT significantly enhanced the patient's response to CCRT, successfully converting an initially inoperable stage IVB disease into an operable condition. The observed downstaging from cT3N2M1 to ypT2NOMO underscores mEHT's potential to achieve remarkable tumor control and improve overall outcomes, even though mEHT was used alongside a suboptimal CCRT dose of 45 Gy administered with palliative intent.

One remarkable feature of mEHT is its ability to trigger an immune response, which enhances the effectiveness of chemotherapy, particularly in reducing lymph node metastasis. By inducing the release of heat shock proteins (HSPs), mEHT generates danger signals that activate the immune system [6]. These HSPs promote the presentation of tumor antigens to immune cells, thereby eliciting a robust anti-tumor immune response [7]. This immune activation contributes to the targeted elimination of cancer cells, including those that have metastasized to lymph nodes [8]. In this case, the CMR of previously affected lymph nodes suggests that the combination of mEHT and CCRT effectively stimulates the immune system to combat metastatic disease.

The complete response (CR) observed in lymph node metastases and tumor downstaging from cT3N2M1 to ypT2NOMO following CCRT combined with mEHT is consistent with prior evidence highlighting the synergistic effects of hyperthermia combined

with chemoradiotherapy for esophageal cancer demonstrated a notably higher CR rate compared to standard CCRT alone (odds ratio 2.00, $p < 0.00001$), indicating that hyperthermia substantially augments tumor radiosensitization and immune activation [9]. Furthermore, previous studies have demonstrated the capacity of mEHT to enhance the efficacy of conventional cancer therapies, including its application in esophageal cancer [10]. These mechanisms are likely integral to the observed therapeutic effect, reinforcing the role of mEHT as a promising adjunct to CCRT in the multimodal treatment of esophageal cancer.

This case further corroborates these findings, suggesting that mEHT could be a valuable adjunct in the treatment regimens of patients with advanced esophageal cancer, especially for those deemed ineligible for surgery. The patient's favorable outcome underscores the importance of exploring multimodal treatment approaches to improve the prognosis for patients with advanced cancers. Future research should prioritize larger cohort studies and randomized controlled trials to validate the efficacy of mEHT when combined with CCRT and other therapeutic strategies.

In conclusion, this case emphasizes the potential of mEHT in advancing the therapeutic response for advanced esophageal cancer. By enabling the conversion of inoperable disease to operable disease, mEHT provides a promising avenue for enhancing patient outcomes. Continued research and clinical trials are essential to fully establish its role in the comprehensive management of esophageal cancer.

Author Contributions

Deng-Yu Kuo: Conceptualization; Resources; Writing original draft. Pei-Wei Shueng: Methodology; Supervision; Writing original draft. Shan-Ying Wang: Supervision; Writing – review and editing. Cheng-Hung How: Supervision; Writing – review and editing. Chen-Xiong Hsu: Conceptualization; Project administration; Supervision; Writing – review and editing.

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This study was conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The present case report was approved by the Research Ethics Review Committee of Far Eastern Memorial Hospital (approval number: 113227-C). Written informed consent was obtained from the patient for the publication of this case report and any accompanying images.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting the findings of this study are available upon reasonable request from the corresponding author. Due to patient privacy regulations and ethical restrictions, the raw clinical data cannot be publicly shared but may be provided in a de-identified format upon institutional review board approval.

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BIOELECTROMAGNETISM FOR CANCER TREATMENT— MODULATED ELECTRO-HYPERTHERMIA

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ABSTRACT

Bioelectromagnetism has the potential to revolutionize cancer treatment by providing a noninvasive, targeted, and potentially more effective complement to traditional therapies. Among bioelectromagnetic techniques, modulated electro-hyperthermia (mEHT) stands out due to its unique characteristics, which have been supported by experimental evidence and clinical validation. Unlike conventional hyperthermia methods, mEHT leverages nonthermal bioelectromagnetic processes, offering a distinct and promising approach in oncology. This differentiation underscores the broader potential for bioelectromagnetic applications in cancer treatment, paving the way for innovative therapeutic strategies.

KEYWORDS: thermal; nonthermal; resonance; modulation; electro-hyperthermia; oncotherapy

I. INTRODUCTION

The potential of bioelectromagnetism to address the therapeutic gap and resistance in current oncological treatments is inspiring. Current oncological treatments often reveal a disparity between the existing state of cancer care and the ideal or optimal standards that could be achieved. Although many cancer treatments can be highly effective, they frequently come with significant side effects. There is a notable gap in managing these side effects and enhancing the overall quality of life for patients undergoing treatment. This gap arises because the growth of cancerous masses tends to be exponential, while the cell distortion associated with malignancy is typically linear or quadratic, as described in radiotherapy [1]. Efforts to compensate for the rapid tumor growth with increased doses are limited by the toxicity of the methods, resulting in a therapeutic gap (Figure 1a). Additionally, cancer cells can develop resistance to therapies over time, further reducing the effectiveness of current treatments and exacerbating the therapeutic gap (Figure 1b). Addressing and overcoming this resistance remains a significant challenge in oncology.

The therapeutic gap manifests in several ways:

- **Access Disparities:** Not all patients have equal access to the latest and most effective therapies. Factors such as a patient's personal health, diet, geographic location, socioeconomic status, and healthcare infrastructure can significantly impact their ability to receive cutting-edge treatments.
- **Customization Limitations:** Although advances in personalized medicine have enabled tailored treatments based on individual genetic profiles, there are still limitations in the extent to which therapies can be customized. This gap may lead to less effective treatments for some individuals.
- **Stage of Diagnosis:** The effectiveness of oncological treatments often depends on the stage at which cancer is diagnosed. Gaps in early detection methods can result in diagnoses at more advanced stages when treatment options may be less effective.
- **Mortality Rates:** Despite new treatments, the mortality rate for some cancers does not decrease significantly. Factors such as an aging population, environmental exposures, and socioeconomic disparities contribute to this persistent issue. Limited access to care, high treatment costs, and geographic disparities further exacerbate mortality rates. Effective

therapies are still lacking for many cancers, highlighting the need for ongoing research to develop new treatments and improve our understanding of cancer mechanisms.

- **Integration of Emerging Technologies:** Emerging technologies such as immunotherapies, targeted therapies, and precision medicine are not always swiftly integrated into standard practice. Bridging this gap involves translating research breakthroughs into clinical practice efficiently and effectively.

Addressing the therapeutic gap necessitates the development of innovative techniques that are minimally toxic, adaptable to the treated subject, complementary to existing therapies, and cost-effective. Ideally, these new technologies should be able to modify the chemical reactions within living organisms without using drugs. Bioelectromagnetism represents a promising candidate for this role, provided its targeting and processes are carefully controlled. Our current objective is to study and explore this potential.

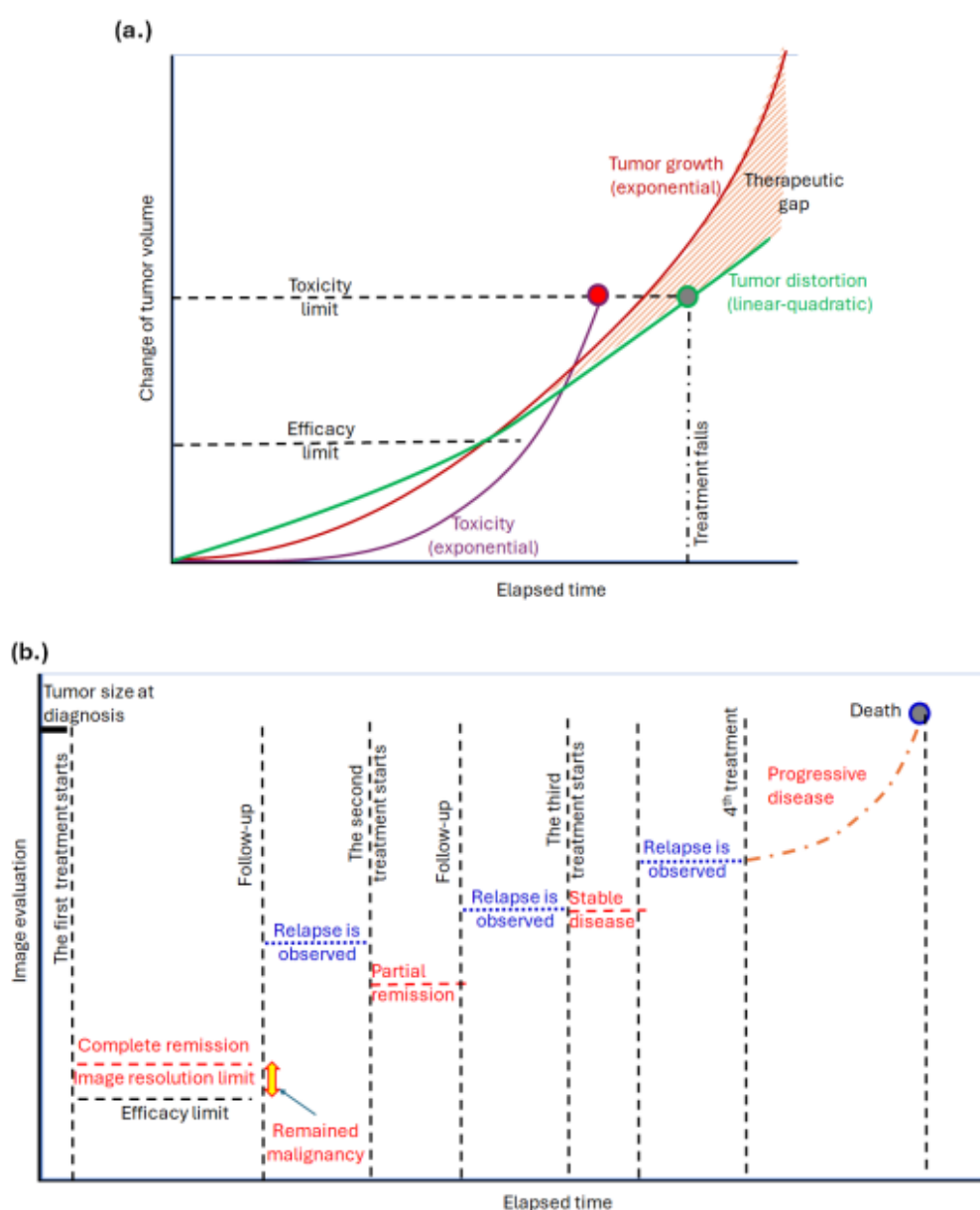


Figure 1. Tumor development. (a) There is a therapeutic gap due to tumors' exponential growth. The toxicity limits the applicable dose. Here, the task of hyperthermia is to extend the limit with extra tumor distortion. (b) The available imaging observes complete remission and later relapses. The subsequent relapses happen due to the exceptional adaptation capability of the tumor cells, which remained in the location under the resolution of the actual imaging. The task of hyperthermia is to introduce new stress and suppress the possibility of adaptation.

2. ELECTROMAGNETIC CHARACTERISTICS OF MALIGNANT CELLS

Cancer cells often exhibit an altered resting membrane potential compared to healthy cells [2]. Cancer cells typically depolarize this potential, influencing various cellular processes, including growth and proliferation [2]. This alteration is partly driven by the Warburg effect, which describes how metabolic reprogramming provides cancer cells with several advantages for growth and survival. The Warburg effect is a metabolic hallmark of cancer cells characterized by increased glucose uptake, lactic acid production, and reduced mitochondrial activity; the cancer cells preferentially undergo aerobic glycolysis, producing lactate even in the presence of oxygen. The resulting increase in extracellular acidity can modify the electrical properties of tissues by altering the ionization state of various molecules and influencing cellular interactions.

Ion channel activity in cancer cells also differs from that in normal cells [3]. The acidic environment resulting from the Warburg effect [4] can affect the function of ion channels and transporters on the cell membrane, contributing to the altered membrane potential. This metabolic shift influences ion channel activity, leading to altered expression of channels that maintain ionic balance, which is crucial for cancer cell survival and proliferation. These changes can impact electrical properties such as membrane resistance and capacitance, with upregulated or mutated ion channels leading to increased ionic currents and altered electrical activity.

Cancer cells and tissues often display different electrical impedances in comparison to healthy counterparts [5]. The accumulation of lactate and other metabolites can influence the conductivity of cancer cells, as acidic conditions may alter the dielectric properties of cells and the surrounding extracellular matrix (ECM), resulting in lower impedance in cancer cells due to differences in cellular structure and density. These changes affect how electrical currents and electromagnetic fields interact with the tissue. The lower impedance facilitates the flow of electric fields and currents through the cancer tissues [6].

The shift to glycolysis in cancer cells also involves changes in the levels and activities of various glycolytic enzymes [7], which can impact the cells' bioelectromagnetic properties. These enzymes may influence intracellular ionic concentrations and electrical signaling pathways [8]. Increased glycolysis generates more metabolic heat due to higher glucose turnover [9], potentially affecting the thermal properties of cancer cells and their interaction with electromagnetic fields, thus influencing the efficacy of electromagnetic therapies.

The ECM in tumors often significantly differs from that in normal tissues [10]. The Warburg effect contributes to changes in the tumor microenvironment, including ECM alterations, which can impact tumor tissue's electrical impedance and conductivity. ECM remodeling in tumors can modify how electrical currents are distributed and how electromagnetic signals are absorbed or

scattered, with changes in the composition and structure of the ECM affecting its conductivity and the transmission of electrical signals through the tissue [11].

Cells emit various electromagnetic signals due to their intrinsic electrical and biochemical activities [12,13], and this inherent noise is characteristic of complex systems. These signals can be categorized based on different aspects of cellular function and their interaction with electromagnetic fields. Due to their deviated metabolism, cancer cells, particularly, can emit distinct bioelectric signals compared to normal cells [14].

Complex, self-organized, self-similar structures characterize the intrinsic organization of living organisms [15]. This self-organized complexity is reflected in the emitted spectrum of chemical activity in cells, manifesting as characteristic fractal dynamics over time [16]. The time–fractal fluctuation of homeostatic processes is associated with noise, where the logarithm of the energy spectral density inversely depends on the frequency of the emitted noise ($1/f$ noise), a characteristic of the homeostatic operation of tissue [17].

3. MODULATED ELECTRO-HYPERTHERMIA (MEHT)

3.1. Mechanisms of mEHT

The key principle of mEHT focuses on the bioelectromagnetic and thermal differences between healthy and malignant cells [18]. Cancer cells exhibit several distinct bioelectromagnetic properties compared to their healthy counterparts. By harnessing well-designed bioelectromagnetic interactions, mEHT can selectively target malignant cells, acting heterogeneously rather than uniformly [18], and effectively complementing conventional oncotherapies. These bioelectromagnetic differences are primarily due to the altered cellular and molecular environment in cancerous tissues, allowing for precise targeting of malignancies.

A critical factor contributing to cancer cells' divergent behavior is their heightened metabolic activity, which fosters their autonomy and disrupts the healthy cellular network. Malignant cells often prefer aerobic glycolysis over oxidative phosphorylation for energy production. This metabolic irregularity is closely linked to cancer cells' electromagnetic properties. The mEHT uses these irregularities to select these cells and destroy them with a synergy of thermal and nonthermal energy from the radiofrequency (RF) electric current [19].

3.2. Technical Implementation of mEHT

The technical realization of mEHT involves a highly precise impedance-matched resonant circuit [20] that adapts to real-time changes in the target tissue. Energy delivery is managed through a dominant electric field in the near-field arrangement of a capacitive coupling, using accurately and dynamically corrected impedance matching [21]. This sensitive tuning technique maximizes treatment efficacy, forming a galvanic-like connection between the electrodes and the patient's body. The patient becomes an integral part of the resonant circuit (Figure 2).

offers diagnostic value [32,33], making mEHT a theranostic technique. The application of 1/f modulation further helps regulate biosystems toward standard homeostasis [34], enhancing the selective destruction of malignant cells.

3.4. Thermal and Nonthermal Processes in mEHT

mEHT leverages the bioelectromagnetic differences between cancerous and healthy cells to achieve targeted treatment. This technique focuses on the unique electrical properties of cancer cells, utilizing bioelectromagnetic interactions to disrupt malignant processes while cooperating with healthy homeostatic mechanisms [35]. These bioelectromagnetic interactions significantly impact the chemistry of malignant tissues, affecting cellular processes, metabolic pathways, and biochemical environments, and could cause cell death. They also influence ion channels, the extracellular matrix (ECM), and protein functions, which can alter tumor growth, response to therapy, and overall cellular behavior.

Electromagnetic fields, particularly those in the radiofrequency range, can cause localized heating of tissues. This thermal effect can modify biochemical reactions by changing tissue temperatures, potentially impacting enzyme activity and metabolic rates. mEHT represents a unique advancement in oncological hyperthermia by combining nonionizing electric fields with thermal effects to target cancer cells more precisely. Unlike conventional hyperthermia, which focuses solely on thermal effects, mEHT integrates thermal and nonthermal processes to enhance treatment efficacy, selecting and destroying the malignancy.

Traditional hyperthermia approaches, rooted in Hippocratic principles, focus on isothermal heating, aiming to evenly distribute temperature across the tumor. However, this approach is flawed because heat disperses from the targeted area, and thermal homeostasis increases blood flow, which may elevate the risk of metastases [36]. The complexity of deep-focused heating reflects the intricate physiological and technical challenges involved. The heterogeneity of living tissues, from intracellular to systemic processes, complicates energy absorption and distribution.

In contrast, mEHT departs from conventional heating methods by focusing its thermal effects on cancer cell membranes and ion channels [37]. It combines thermal and nonthermal impacts, similar to the synergy between conventional hyperthermia and ionizing radiotherapy. The thermal effects provide a general background for optimizing the nonthermal processes. The thermal background determines, in general, the chemical reaction rates according to Arrhenius law. The chemical reactions caused by nonionizing (bioelectric fields), ionizing (radiotherapy), and chemical changes (chemotherapy) all change their rate and efficacy by the thermal conditions (Figure 3). This approach leverages the heterogeneity of tissue thermal and electrical properties to selectively target and eliminate malignancies [37].

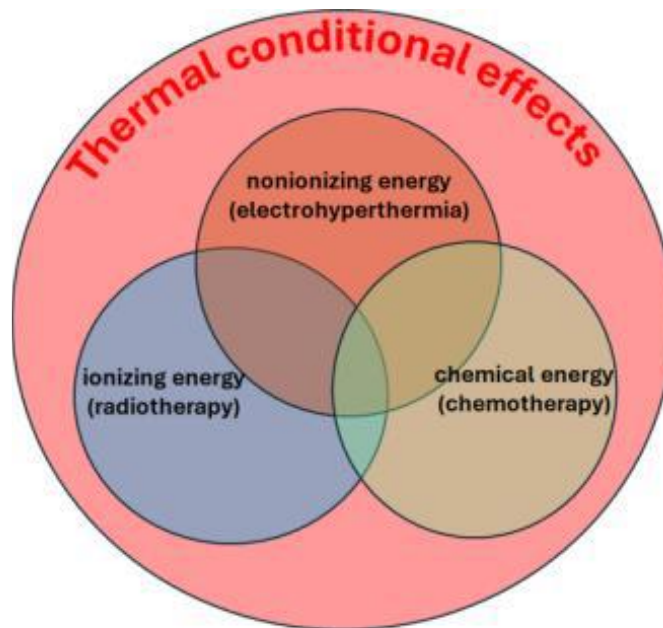


Figure 3. The thermal background guarantees the optimization of the nonthermal processes.

Cancer cells exhibit distinct temperature characteristics compared to healthy cells due to their altered metabolism and blood supply. Cancer cells generally have a higher temperature than normal cells. This is primarily attributed to their increased metabolic activity. While generally warmer, a tumor's temperature can vary depending on factors like tumor type, size, location, and blood supply. Tumors with poor blood supply may exhibit lower temperatures in certain areas, while well-vascularized tumors tend to have more uniform temperature distribution. Cancer cells can be more sensitive to temperature changes than normal cells. Some cancer cells are more susceptible to heat-induced damage, making hyperthermia a potential treatment option for certain cancers. Cancer cells have a much higher density of chaperone proteins (mostly HSPs) than healthy cells, so by extra heat, they develop relatively fewer extra chaperones. In contrast, healthy cells massively increase their protective chaperone content. This reaction difference is one of the factors of the higher sensitivity of cancer cells to additional heat. Conversely, some cancer cells can adapt to temperature fluctuations, which may contribute to their survival and resistance to heat treatment. Understanding the temperature characteristics of cancer cells is crucial for developing and optimizing cancer therapies. Hyperthermia aims to selectively heat and destroy cancer cells while minimizing damage to healthy tissues. The temperature within the tumor microenvironment can influence various aspects of cancer progression, including cell growth, survival, angiogenesis, and immune response. While temperature can serve as a therapeutic target, it is crucial to consider the complex interplay between temperature and other factors in cancer development and treatment. Understanding these complex interactions is essential for developing more effective cancer treatments.

The nonthermal effects of mEHT involve altering ion channel activity and intracellular ion concentrations, which can impact cellular processes such as signal transduction, enzyme activity, and pH levels. The primary nonthermal processes make a broad range of interactions, which could be modified with the applied electromagnetic techniques of mEHT.

A significant process is that electromagnetic fields can nonthermally influence the activity of ion channels and transporters in cancer cells. Changes in ion channel function can alter the intracellular concentrations of ions such as calcium, sodium, and potassium. This affects various cellular processes, including signal transduction, enzyme activity, and cellular pH, which can indirectly modify the cell's biochemical environment. The main deviation of mEHT from conventional heating techniques is that it helps and does not impede natural homeostatic actions. Constrained heating may destroy the tumor but can also cause a large attack on homeostatic regulation, which tries to compensate for the intensive local changes. So, homeostasis is loaded with two obstructions: cancer and aggressive heating (Figure 4a). The thermal and nonthermal synergy, which is optimized with a modulated RF signal in the mEHT method, helps the homeostatic regulation, induces immunogenic effects, and so extends the local effects to the whole body (Figure 4b).

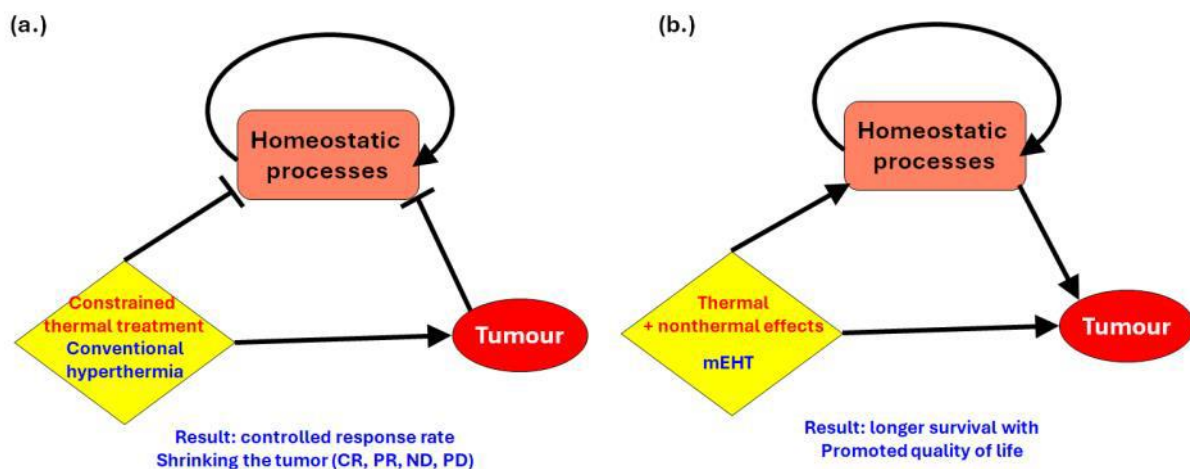


Figure 4. The difference between conventional hyperthermia and mEHT concerning natural homeostasis. (a) The effect of traditional hyperthermia. It works with thermal cell killing and has a reasonable remission rate. (b) mEHT works in harmony with homeostatic processes and elongates survival time with an increase in quality of life.

mEHT alters the membrane potential of cancer cells and can affect cellular excitability and the activity of ion pumps and channels, leading to changes in intracellular ionic balance and affecting metabolic processes. Exposure to electromagnetic fields of mEHT can influence metabolic pathways in cancer cells. The mEHT-induced electromagnetic fields might impact glycolysis, oxidative phosphorylation, or lipid metabolism. These changes can influence metabolic pathways, potentially shifting the production of metabolic byproducts like lactate or reactive oxygen species (ROS). ROS can damage cellular components and contribute to tumor progression or therapy sensitivity.

mEHT also affects the ECM, altering the biochemical environment of malignant tissues. ECM composition and structure changes can influence cell adhesion, migration, and signaling pathways, potentially modifying the tumor microenvironment. Additionally, the electric field of mEHT can impact extracellular pH, affecting tumor enzyme activities and biochemical reactions. Enhanced cellular permeability due to the electric field can alter the transport of chemicals and metabolites, influencing intracellular concentrations and biochemical reactions. It can also affect protein conformation and function, impacting enzyme activity, protein interactions, and cellular signaling. Such alterations can lead to changes in metabolic pathways and cellular responses.

Notably, mEHT's electromagnetic fields may influence gene expression by affecting transcription factors or epigenetic modifications [38]. This can alter protein synthesis related to metabolism, signaling, and cell growth, impacting overall cellular chemistry.

mEHT targets the membrane rafts of malignant cells [39,40]. It heats the transmembrane proteins of these cells similarly to nanoparticle heating [19], but without the need for artificial nanoparticles [41]. This process utilizes the differences in thermal and electromagnetic properties between malignant and healthy cells, taking advantage of the higher electric conductivity and disordered tumor microenvironment (TME) in malignant cells compared to healthy ones.

mEHT uniquely employs modulation of the radiofrequency (RF) signal. This low-frequency amplitude modulation promotes the polarization and excitation of transmembrane proteins, driving chemical reactions to induce an apoptotic process and trigger intracellular signaling pathways for immunogenic cell death. This modulation addresses the challenge of combining high-frequency RF for nano-selection with low-frequency modulation for molecular polarization. The precise targeting is based on the thermal and electric heterogeneity of living tissues, focusing on the specific characteristics of cancer cells.

Various signal pathways ensure the apoptotic processes. The external RF excites the TRAIL–FASD–FAS complex, starting an external trigger of the caspase 8 → caspase 3 → apoptosis [42] molecular excitation line. Involving mitochondria by heat, an electric field, and Bid protein, an intrinsic apoptotic pathway is formed through Bax→Cytochrome C→Caspase 9→Caspase 3→apoptosis [42] and a caspase-independent path, mitochondria → AIF → apoptosis [43]. The inhibitory, antiapoptotic XIAP protein is blocked by Septin 4 [44] and SMAC/Diablo proteins [45].

3.5. Immune Effects of mEHT

Cancer therapies aim to destroy cancer cells while restoring healthy conditions. Cell death is possible, like apoptosis, necrosis, autophagy, necroptosis, ferroptosis, and pyroptosis. Inflammatory cell death involves types like pyroptosis and necroptosis, which are programmed forms of cell death that activate inflammatory pathways. Uncontrolled necrosis can also cause inflammation due to the chaotic release of cell contents. In contrast, apoptosis is a non-inflammatory, controlled process where cells die in an orderly manner and are removed by phagocytes without causing inflammation. Non-inflammatory cell death maintains tissue homeostasis and minimizes collateral damage.

Immunogenic cell death (ICD) is a form of cell death that activates the immune system. It is characterized by releasing damage-associated molecular patterns (DAMPs) that alert and activate immune responses. mEHT induces ICD by producing DAMPs [46], which trigger immune responses in an organized manner through harmonized thermal and nonthermal effects. These DAMPs provide information about the malignancy [47], enabling antigen-presenting cells to produce tumor-specific killer and helper T cells that recognize and destroy tumor cells through natural immune surveillance. The subsequent steps of the process are summarized in Figure 5.

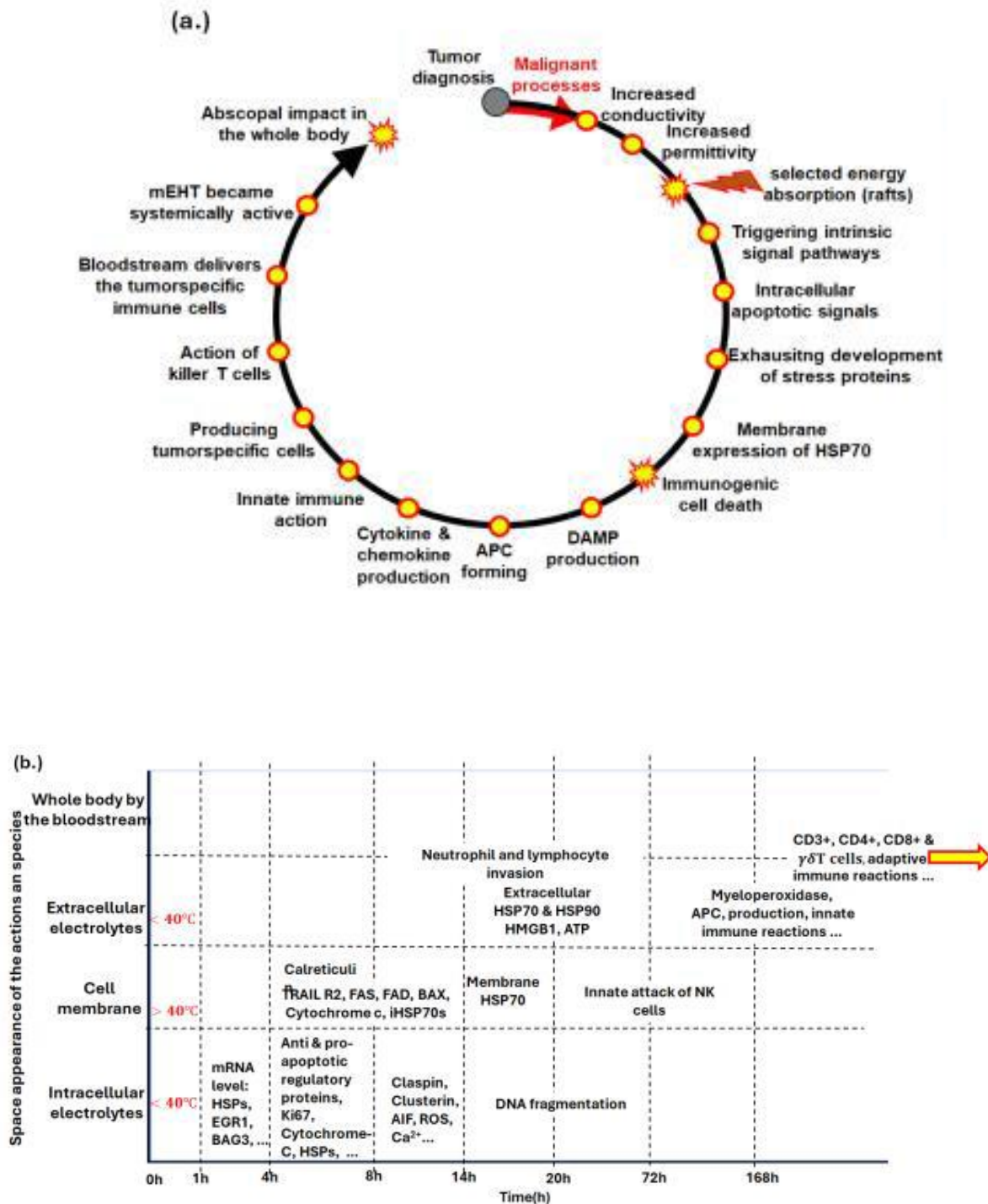


Figure 5. The immunogenic processes ignited by mEHT. (a) The steps of immunogenic development after mEHT treatment. (b) The molecular effects with elapsed time. The yellow arrow is intended to show that the immunogenic process continues even after 168 h.

Preclinical research is a multi-step process. It begins with the concept that mEHT's heterogeneous energy absorption enhances antitumor effects by inducing the exhaustion of heat-shock proteins (HSPs) in cancer cells [48]. Released HSPs and other DAMPs, which are designed in proper order in space and time [47], facilitate antigen presentation for tumor-specific T CD8+ cells [49], boosting the immune response against the tumor. This prepares the immune system to recognize malignant

cells throughout the body, enabling systemic antitumor immune activity and potentially destroying distant micro- and macrometastases (abscopal effect) [50]. Memory T cells retain information about the tumor, making subsequent tumor recurrence unlikely, akin to a vaccination against the cancer. This vaccination approach is patented [51].

The theoretical consideration and concept design are followed by in silico research and phantom measurements to verify thermal effects. In vitro experiments are conducted next, although they lack the complexity of living organisms. The research progresses to in vivo studies, providing feedback and refining the concept until it demonstrates sufficient accuracy, safety, and efficacy for clinical trials [52]. Preclinical studies have validated the selection process [53], confirmed immunogenic effects [54], and demonstrated the development of DAMPs [55] and ICD [49] and the abscopal effect through immune activation of killer T-cells [56]. The comparison shows the superiority of mEHT over conventional hyperthermia [42,57,58].

The addition of nonthermal effects to conventional thermal processes has been validated through several experimental and preclinical studies [42,52,59]. Phantom experiments verified thermal effects and selective heating [60]. Various laboratories measured the apoptosis and the synergetic combination of the thermal and nonthermal effects [61,62]. The experimental conclusion was that mEHT produces massive apoptosis. An overall review of the preclinical verification in vitro and in vivo [63] and immunogenic proofs [64] were published elsewhere.

Clinical efficacy validation was provided for different tumors [65,66]. See Table 1. As clinically reviewed in detail elsewhere [65,66], it was proven how clinical studies validate mEHT's efficacy. A Phase III study showed a significant increase in survival time for patients with advanced cervical cancer [67], with observed abscopal effects [68] and improved quality of life [63]. Phase II trials have reported significant improvements with mEHT for severe cancers [66], including the frequent and hard-to-be-successful treatments for localizations such as pancreas [64,69], glioblastoma [70–72], non-small-cell lung cancer [73], small-cell lung cancer [74], rectal cancer [75], pain of bone metastases [76], and pediatric DIPG [77]. Additional studies and case reports support the effectiveness of combining mEHT with immune checkpoint inhibitors [78,79] and supportive therapies [80].

Table 1. A selection of the clinical studies using the mEHT method.

No.	Tumor Site	No. Pts.	Treatment Used	Results	Ref.
1	Advanced relapsed gliomas	12	mEHT + RT + ChT	RR = 25%. PFS = 10 m. OS = 9 m, 1yS = 25%.	Fiorentini et al., 2006 [81]
2	Mostly advanced brain gliomas	140	mEHT + RT + ChT	OS = 20.4 m. QoL: safe and well tolerated.	Sahinbas et al., 2007 [82]
3	High-grade gliomas	179	mEHT + RT + ChT	KS > 50%, AST: No. = 53, 1yS = 100%, 5yS = 51%, OS = 38.2 m, events: 19.8%, GBM: No. = 126, 1yS = 92%, 5yS = 38%, OS = 20.3 m, events: 26.4%, QoL: improved.	Hager et al., 2008 [70]
4	Relapsed gliomas	149	mEHT + RT + ChT (BSC)	Double arm, palliation setup. AST: No. = 38, mEHT No. = 28, RR: mEHT = 48%, Ctrl = 10%, 5yS: mEHT = 83%, Ctrl = 25%, OS: mEHT = 16.5 m, GBM: No. = 111, mEHT No. = 24, RR: mEHT = 29%, Ctrl = 4%, 5yS: mEHT = 3.5%, Ctrl = 1.2%, OS: mEHT = 14 m. QoL: improved.	Fiorentini et al., 2018 [71]

Table 1. Cont.

No.	Tumor Site	No. Pts.	Treatment Used	Results	Ref.
5	High-grade gliomas	20	mEHT + RT	PFS: 1.5–3 m, QoL: No severe side effects, well tolerated.	Solodkiy et al., 2021 [83]
6	Relapsed gliomas	164	mEHT + RT + ChT	Double arm. RR: mEHT = 41.4%, Ctrl = 33.4%, OS: mEHT = 15 m, Ctrl = 12 m. 1yS: mEHT = 55%, Ctrl = 15%, QoL: improved.	Fiorntini et al., 2020 [84]
7	Recurrent gliomas	76	ChT + mEHT	Comparison to ddTMZ historical ctrl. No.: mEHT + ddTMZ = 58 mEHT = 18, Ctrl = 79. RR(incl.SD): mEHT = 55%, mEHT + ddTMZ = 53%, events: mEHT = 67%, mEHT + ddTMZ = 67%, OS: mEHT = 14.8 m, mEHT + ddTMZ = 20.8 m, 1yS: mEHT = 22.6%, mEHT + ddTMZ = 29.5%, 5yS: mEHT = 0%, mEHT + ddTMZ = 13.5%, cost-effectiveness analysis: ddTMZ + mEHT is cost-effective.	Roussakow, 2017 [85]
8	Advanced GBM	60	mEHT + RT + ChT	PFS = 13 m. Follow-up = 17 m, OS ≈ 30 m. QoL: no added toxicity.	Van Gool et al., 2018 [86]
9	Ovarian cancer	12	ChT ± mEHT	Double combined arms: PLX, CIS. PFS: mEHT + PLX = 3 m, mEHT + CIS = 6.8 m, OS: mEHT + PLX = 11.5 m, mEHT + CIS ≈ 20 m. DLT = not observed, mEHT is feasible, safe.	Kim et al., 2021 [87]
10	Recurrent, progressive ovarian cancer	19	mEHT	PFS = 4 m, OS = 8 m, events = 18. DLT = not observed. mEHT is feasible, safe.	Yoo et al., 2019 [88]
11	Advanced cervical cancer	95	CCRT ± mEHT	No.: CCRT = 53, mEHT + CCRT = 42, NED: CCRT = 68%, mEHT + CCRT = 83%. 5yDFS: CCRT ≈ 75%, CCRT + mEHT ≈ 80%, 5yS: CCRT ≈ 81%, CCRT + mEHT ≈ 79%, mets.5yDFS: CCRT ≈ 84%, CCRT + mEHT ≈ 62%, 5yOS: CCRT ≈ 73%, CCRT + mEHT ≈ 78%.	Lee et al., 2023 [89]
12	Advanced cervical cancer ±HIV	202	CCRT ± mEHT	Prospective, double arm. No.: CCRT = 101, mEHT + CCRT = 101. Alive at 6 m after treatment: CCRT = 82.2%, CCRT + mEHT = 87.1%, LDC(RR): CCRT = 24.1%, CCRT + mEHT = 45.5%, LSFS: CCRT = 19.8%, CCRT + mEHT = 38.6%. 5yS: CCRT26%, CCRT + mEHT = 33%, DFS: CCRT = 14%, CCRT + mEHT = 32%. No significant differences in acute adverse events or quality of life between the groups.	Minnaar et al., 2022 [67]
13	Advanced cervical cancer, ±HIV	100	Phase III (RT + ChT ± mEHT)	No. = 100 (preliminary data). A positive trend in survival and local disease control by mEHT. QoL: no significant differences between the groups.	Minnaar et al., 2016 [90]
14	Advanced cervical cancer	72	mEHT + RT + ChT	RR = 73.5%; SD = 14.7%. Adding mEHT increased the QoL and OS compared to historical data.	Pesti et al., 2013 [91]
15	Advanced cervical carcinoma	20	mEHT + RT + ChT	mEHT increases the peri-tumor temperature and blood flow in human cervical tumors, promoting radiotherapy + chemotherapy.	Lee et al., 2018 [92]
16	Advanced cervical carcinoma	38	mEHT + ChT	Double arm. No. ChT = 20, ChT + mEHT = 18. RR: ChT = 40%, ChT + mEHT = 72.2%. 2yS: ChT ≈ 50%, ChT + mEHT ≈ 90%.	Lee et al., 2017 [93]

Table 1. Cont.

No.	Tumor Site	No. Pts.	Treatment Used	Results	Ref.
17	Advanced cervical carcinoma, $\pm$ HIV	206	Phase III (CCRT $\pm$ mEHT)	Abscopal effect: CCRT = 5.56%, CCRT + mEHT = 24.07%, measured with complete metabolic response by PET of all sites of disease.	Minnaar et al., 2020 [68]
18	Advanced cervical carcinoma, $\pm$ HIV	206	Phase III (CCRT $\pm$ mEHT) [toxicity and quality of life]	mEHT addition does not increase the toxicity, QoL: improvement in social, emotional, and physical function in CR significantly higher with mEHT addition ($p < 0.02$).	Minnaar et al., 2020 [63]
19	Advanced cervical carcinoma	202	mEHT + RT + ChT	Six-month local disease-free survival (LDFS) = 38.6% for mEHT and LDFS = 19.8% without mEHT ($p = 0.003$). Local disease control (LDC) = 45.5% with mEHT, LDC = 24.1% without mEHT; ($p = 0.003$).	Minnaar et al., 2019 [94]
20	Advanced, recurrent, or metastatic breast cancer	10	mEHT + ChT/RT.	No. = 10 (all ER-positive, 1 HER2), ORR = 55%.	Nagata et al., 2021 [95]
21	Advanced NSCLC	97	mEHT + RT + Vit.C	OS: RT + Vit.C = 5.6 m, mEHT + RT + Vit.C = 9.4 m, PFS: mEHT + RT + Vit.C = 3 m, RT + Vit.C = 1.85 m ($p < 0.0001$).	Ou et al., 2020 [80]
22	NSCLC	311	mEHT + RT + ChT	No. RT + ChT = 53 (historical), RT + ChT + mEHT = 258. OS: RT + ChT = 14 m, RT + ChT + mEHT = 15.8 m. Advanced subgroup: No. RT + ChT = 43 (historical), RT + ChT + mEHT = 140. OS: RT + ChT = 11 m, RT + ChT + mEHT = 14.7 m.	Szasz, 2014 [96]
23	Advanced NSCLC adenocarcinoma	4	mEHT + ChT	Survived for more than 2 years with combined therapy. No complete remission was achieved.	Lee et al., 2015 [97]
24	Advanced hepatocellular carcinoma	21	mEHT + Sorafenib	RR = 55% (no CR was observed), 6 mPFS = 38%, PFS = 5.2 m, OS = 10.4 m.	Gadaleta-Gadaleta et al., 2014 [98]
25	Advanced rectal cancer	76	mEHT + RT + ChT	RR = 33.3%, Gr.I thermal toxicity = 26.7%.	You et al., 2020 [99]
26	Rectal cancer	120	mEHT + RT + OP	No.: RT + OP: 58, RT + OP + mEHT = 62. Downstaging: RT + OP: 67.2%, RT + OP + mEHT = 80.7%. Gastrotoxicity: RT + OP: 87.9%, RT + OP + mEHT = 64.5%, 2yDFS: RT + OP: 79%, RT + OP + mEHT = 96%.	Kim et al., 2021 [75]
27	Pancreas	73	ChT + mEHT	During the initiation of mEHT, immune markers stabilize with the treatment, but progressive disease erodes this positive effect over time. Long-time follow-up shows a significant increase in the WBC, neutrophil, and granulocyte counts, and an increase in CRP, NLR, and GLR was observed.	Dobos et al., 2024 [100]
28	Pancreas	217	ChT $\pm$ mEHT	No.: ChT = 128, ChT + mEHT = 89. OS: ChT = 9 m, ChT + mEHT = 20 m. RR: ChT = 24%, ChT + mEHT = 45%. PD: ChT = 31, ChT + mEHT = 4.	Fiorentini et al., 2023 [69]
29	Pancreas	158	ChT + mEHT	No.: ChT = 100, ChT + mEHT = 58. OS: ChT = 11.02 m, ChT + mEHT = 19.5 m. RR: ChT = 58%, ChT + mEHT = 95%. Toxicity did not differ in the two groups, only 8 pts. had Gr.1–2 burns.	Fiorentini et al., 2021 [64]

Table 1. Cont

No.	Tumor Site	No. Pts.	Treatment Used	Results	Ref.
30	Pancreas ductal adenocarcinoma	78	mEHT + ChT	Non-operable patients. No.: ChT = 39, ChT + mEHT = 39. OS: ChT = 14.19 m, ChT + mEHT = 16.96, PFS: ChT = 8.53 m, ChT + mEHT = 11.87, 1yS: ChT = 16%, ChT + mEHT = 26%. 2yS: ChT = 5%, ChT + mEHT = 6%, 1yPFS: ChT = 7%, ChT + mEHT = 11%.	Petenyi et al., 2021 [101]
31	Advanced pancreas carcinoma	106	mEHT + RT + ChT	After 3 m, PR = 22 (64.7%), SD = 10 (29.4%), PD = 2 (8.3%) with mEHT after 3 m of the therapy. In the group without mEHT at the same time, PR = 3 (8.3%), SD = 10 (27.8%), PD = 23 (34.3%). The median OS = 18 m with mEHT and OS = 10.9 m without mEHT.	Fiorentini et al., 2019 [102]
32	Advanced pancreas carcinoma	133	mEHT + RT + ChT	Two centers. No.: PFY = 26, HTT = 73, Ctrl. = 34. Conventional therapies failed. Distant metastases: PFY = 59%, HTT = 88%. OS: PFY = 12 m, HTT = 12.7 m, Ctrl. = 6.5 m. 1yS: PFY = 46.2%, HTT = 52.1%, Ctrl. = 26.5%. QoL was improved.	Dani et al., 2008 [103]
33	Metastatic pancreas carcinoma	26	mEHT + ChT	RR = 71%, PFS = 3.9 m. OS = 8.9 m.	Volovat et al., 2013 [104]
34	Gastrointestinal cancer	49	mEHT + CCRT + IMT	OS: CRC and GC ≈ 20 m CCC and GC ≈ 10 m. The IMT + mEHT had the most positive effect on overall survival (HR: 0.3055; $p = 0.0260$), IL-2 and low-dose ipilimumab showed a positive tendency.	Kleef et al., 2023 [105]
35	Advanced gastric cancer	24	mEHT + MSCT + HBOT	CR = 88.0%, follow-up = 23.9 m. OS = 39.5 m, PFS = 36.5 m.	Iyikesici et al., 2020 [106]
36	Advanced abdominal soft-tissue sarcoma	24	mEHT + ChT	RR = PR + SD = 88%	Volovat et al., 2014 [107]
37	Various sites	30	RT + mEHT	CR = 22.2%, PR = 55.5%, SD = 14.8%, PD = 7.4%.	Chi, 2020 [108]
37	Various sites	131	IMT + mEHT	RR = 31.3%, PFS = 10 m, 1yS = 66.5%, 2yS = 36.6%.	Kleef et al., 2020 [79]
39	Various sites (advanced, metastatic)	784	mEHT + RT + ChT + OP	GBM (n = 36, OS = 2.2y); breast cancer (n = 72, OS = 5y), coloncancer (n = 79, OS = 4y) NSCLC (n = 54, OS = 1.5y), PC (n = 27, OS = 1.1y), soft-tissue sarcoma (n = 16, OS = 3.1y), melanoma (n = 12, OS = 2.6y); ovarian cancer (n = 33, OS = 3.5y), kidney cancer (n = 13, OS = 4.1y), prostate cancer (n = 25, OS = 8.3y), rectal cancer (n = 13, OS = 4.0y), uterine cancer (n = 13, OS = 4.0y), head and neck cancer (n = 13, OS = 4.0y), lymphoma (Hodgkin and non-Hodgkin) (n = 13, OS = 4.0y), cholangiocarcinoma (n = 13, OS = 4.0y).	Parmar et al., 2020 [109]
40	Primary, recurrent, and metastatic sarcomas	13	mEHT + RT + ChT	Cases. Good response.	Jeung et al., 2015 [110]

Table 1. Cont.

No.	Tumor Site	No. Pts.	Treatment Used	Results	Ref.
41	Pelvic and spinal bone metastases	61	RT + mEHT	Pain intensity: before = 37, after = 13. Frequency of occurrence of BPE: before = 33, after = 13. Relative frequency taking painkillers: before = 66, after = 62. Duration of pain release 1.5 weeks longer with RT + mEHT than with RT of mEHT alone.	Kim et al., 2024 [76]
42	Peritoneal carc. with malignant ascites	260	mEHT + TCM	No.: mEHT + TCM = 130, TCM = 130. RR: mEHT + TCM = 77.69%, TCM = 63.8 ($p = 0.005$). KPS: mEHT + TCM = 49.23%, TCM = 32.3% ($p < 0.05$).	Pang et al., 2017 [111]
43	Metastatic cancers (colorectal, ovarian, breast)	23	mEHT + ChT	RR: 85.7% (80 days), 72.2% (160 days). OS (mean) = 497 days, PFS (mean) = 339 days.	Ranieri et al., 2017 [112]
44	Metastatic/recurrent cancers (different types)	33	mEHT + RT	RR = 87.9%. Autoimmune toxicity = 9.1% (abscopal effects were observed).	Chi et al., 2020 [78]

CCRT = concurrent chemoradiotherapy, DFS = median disease-free survival, PFS = median progression-free survival, OS = median overall survival, RR = response rate, CR = complete remission, LDC = local disease control, LDFS = local disease-free survival, 5yS = five-year survival, 1yS = one-year survival, Ctrl. = control, ddTMZ = dose-dense temozolomide, CCC = cholangiocellular cancer, GC = gastric cancer, EC = esophageal cancer, CRC = colorectal cancer, PC = pancreatic cancer, MSCT = metabolically supported chemotherapy, HBOT = hyperbaric oxygen therapy, BTP = breakthrough pain, TCM = traditional Chinese medicine, KPS = Karnofsky performance score.

4. CONCLUSIONS

Depending on electromagnetic exposure's strength, frequency, and duration, bioelectromagnetic interactions can influence molecular reactions. mEHT, in particular, offers a promising avenue for cancer treatment by selectively targeting malignant cells through nonthermal electromagnetic actions. These interactions can open and close membrane ion channels, alter membrane potentials, and impact cellular processes such as signal transduction and protein function, potentially influencing gene expression and chemical reactions at the molecular level.

While these effects are supported by theoretical and experimental studies, as well as preclinical and clinical validation, the full extent of bioelectromagnetic interactions on molecular reactions in living organisms remains an active research area. The potential of mEHT to extend the effects of local treatment to systemic levels is a particularly exciting area of exploration. With its superior effectiveness, ease of use, and high safety record, mEHT represents a promising advancement in oncotherapy, warranting further investigation.

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Conflicts of Interest:

The author is one of the developers of the mEHT method.

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MODULATED ELECTRO-HYPERTHERMIA THERAPY COMBINED WITH KOREAN MISTLETOE EXTRACT TREATMENT EXERTS A STRONG ANTI-TUMOR ACTIVITY BY ENHANCING CELLULAR AND HUMORAL IMMUNE RESPONSES IN MICE

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Electro-hyperthermia therapy (EHT) has been known to cause temperature-dependent cell death and enhance the effects of conventional antitumor treatments, such as chemotherapy and radiotherapy. Furthermore, EHT modulates the innate and adaptive immune systems. Mistletoe is one of the most broadly studied complementary and alternative therapeutic agents for cancer treatment due to its ability to stimulate the immune systems. This study aimed to investigate the effects of EHT and mistletoe therapy combination on immune responses. Tumors induced by B16-BL6 melanoma cells were treated twice with modulated EHT (mEHT) (43°C for 10 or 20 min) and with intravenous injection of a Korean mistletoe extract (KME). We examined the level of interferon (IFN)- γ , granzyme, interleukin (IL)-2, IL-10, and tumor-specific antibodies using enzyme-linked immunosorbent assay methods to further study the immunological responses in the combination of mEHT and KME. Additionally, cytotoxic T lymphocyte (CTL) activity is investigated. In this study, we revealed a significant anti-tumor immunological activity elevation in tumor-bearing mice by combined mEHT and KME therapy. Specifically, the combination of mEHT and KME treatment was effective in inhibiting tumor growth in mice. The combination treatment elicited CTL immune response and increased IFN- γ and granzyme secretion. Particularly, the co-treatment appeared to efficiently suppress the immune signal related to tumor-associated macrophage differentiation. Importantly, tumor cellspecific antibodies could be induced in mice after mEHT-treated tumor cell immunization, which represent a promising cancer vaccine strategy. Thus, our results indicate the therapeutic actions of KME as a feasible partner of mEHT, suggesting its potential candidate for cancer immunotherapy.

Abbreviations:

APC,	Antigen-presenting cell;
CTL,	Cytotoxic T lymphocyte;
EHT,	Electrohyperthermia therapy;
ELISA,	Enzyme-linked immunosorbent assay;
HSP,	Heat shock protein;
KME,	Korean mistletoe extract;
NK,	Natural killer;
PBS,	Phosphate-buffered saline;
QOL,	Quality of life;
RF,	Radio-frequency;
TAM,	Tumor-associated macrophage

KEYWORDS

Electro-hyperthermia; mistletoe therapy; B16-BL6 melanoma; tumor-specific antibodies; cancer vaccine

INTRODUCTION

Electro-hyperthermia therapy (EHT) is a form of cancer therapy that induces temperature-dependent damage to cell proteins and structures that leads to cell death (van der Zee 2002). Various changes occur inside the cells when tumor cells are heated at 41°C – 42°C, which then renders the cancer cells more vulnerable to other anti-tumor treatments (Hildebrandt et al. 2002).

Therefore, EHT is commonly used with other cancer treatment, such as chemotherapy and radiation therapy (Wust et al. 2002). Modulated EHT (mEHT), also known as oncothermia, is a hyperthermia device that is used for treating various cancer types. Malignant lesions are preferentially heated relative to adjacent normal tissues in clinical mEHT application (Andocs et al. 2009; Hegyi et al. 2013a, 2013b). mEHT, combined with classical or targeted chemotherapeutic agents, has been demonstrated to be markedly effective in clinical trials (Wismeth et al. 2010; Gadaleta-Caldarola et al. 2014).

Whole-body mEHT at fever range temperatures has shown general immune system stimulation in the hosts (Evans et al. 2015), and local mEHT of tumors elicit anti-tumor immunity (Wang et al. 2001a; Skitzki et al. 2009). The mechanisms underlying the heat-induced stimulation of immune response include heat shock protein (HSP) generation and antigen-presenting cell (APC) activation (Skitzki et al. 2009). HSPs that are produced in response to hyperthermia treatment act as a signal to elicit immune responses (Wang et al. 2001a; Schmitt et al. 2007). HSP-derived tumor peptide complex is effectively endocytosed by APCs through several HSP receptors, and it activates dendritic cells, which are the most potent APCs (Matsumoto et al. 2011). Notably, mEHT treatment has been reported to create a more favorable microenvironment for immune response induction than conventional hyperthermia, thereby inducing tumor cell apoptosis (Cha et al. 2015; Tsang et al. 2015a).

Mistletoe is a semi-parasitic plant that has been utilized as a traditional medicine in many countries to treat various human illnesses (Adesina et al. 2013; Moghadamtousi et al. 2014). Mistletoe is widely used in Europe where diverse mistletoe extracts are manufactured as injectable anti-cancer drugs or supplements (Galun et al. 2015). The anti-cancer effect of Korean mistletoe (*Viscum album coloratum*) has been widely studied in the past few decades. Reportedly, the Korean mistletoe extract (KME) enhances the host defense system against tumors via macrophage and natural killer (NK) cell activation, which indicates that KME is a potential anti-cancer immunotherapy agent (Yoon et al. 1998b; Yoon et al. 2001; Yoon et al. 2003; Kim et al. 2014).

This study aimed to delineate the anti-tumor immune response elicited by the combination of mEHT and KME treatments. The combined therapy effectively inhibited tumor growth by significantly increasing the T lymphocyte activity, interferon (IFN)- γ , and granzyme secretion. Consistently, the combined treatment of mEHT and KME inhibited tumor-associated macrophage (TAM) differentiation. Furthermore, mEHT-treated tumor cells were observed to be potentially useful as a tumor vaccine.

MATERIALS AND METHODS

Cell culture

The mouse melanoma cell line B16-BL6 and colon carcinoma cell line 26-M3.1 were grown in DMEM (GIBCOBRL; Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum and 0.03% l-glutamine. Cultures were maintained at 37°C in a humid atmosphere of 95% air and 5% CO₂.

Treatment with mEHT

The Lab-EHY device (OncoTherm, Hungary) was used for mEHT treatment in vitro and in vivo following the instruction manuals (<https://www.oncotherm.com/web/phy/EHY%20Lab.pdf>). In the case of mEHT, the Lab-EHY machine was set up at each temperature. Tumor cells and tumors were also heated with water bath.

Preparation of KME

The leaves of Korean mistletoe were grounded and homogenized with 10 volumes of distilled water. The suspension was stirred overnight at 4°C and centrifuged at 15,000×g for 30 min. Then, the supernatant was filtered and lyophilized. The obtained brown powder was dissolved in phosphate-buffered saline (PBS) and stored at 4°C until use.

Animals and tumor induction

Female C57/BL6 mice of 5–7 weeks old were used for the experiments (DBL, Incheon, Republic of Korea). The animals were housed (4–5 mice per cage) in a temperature-controlled room. After 7 days of acclimatization, each mouse was intradermally inoculated on the back with 1×10^5 B16-BL6 melanoma cells in 0.05 ml of saline. Mice were anesthetized by intraperitoneal injection of 250 µl of solutions containing 1:1 mixture of avertin stock and 5-fold diluted Rompun. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Dongguk University (IACUC No. 2021-O20-3).

Cytotoxic T lymphocyte (CTL) assay

CTL assay was performed as previously described (Rohrer et al. 2006). Briefly, spleen cells were isolated from control and treated mice and were minced with a homogenizer and washed with PBS. After removing the erythrocytes, the splenocytes were cultured with B16-BL6 melanoma cells in a 37°C incubator under humidified 5% CO₂ atmosphere. Cytotoxicity assays were done by incubating spleen cells (effector cells [E]) with 1×10^5 melanoma cells (target cells [T]) at an E:T ratio of 100:1, 50:1, or 25:1. The specific cytotoxicity was measured using the LDH assay kit (Promega, Madison, WI, USA) after 6 h of incubation.

Measurement of tumor-specific antibody titers

Mice were immunized by subcutaneous injection of 2×10^5 tumor cells. The serum antibody titers from the immunized mice were measured by ELISA. Briefly, approximately 1×10^5 tumor cells were plated on a flat-bottomed microtiter plate and fixed with 70% methanol. The plate was then blocked with 1% skim milk and washed with PBS. Serum was diluted 100-fold, added to the wells, and incubated for 2 h. Antibody titers were measured using HRP-conjugated rabbit secondary antibody to mouse immunoglobulin (Ig)G (R&D Systems).

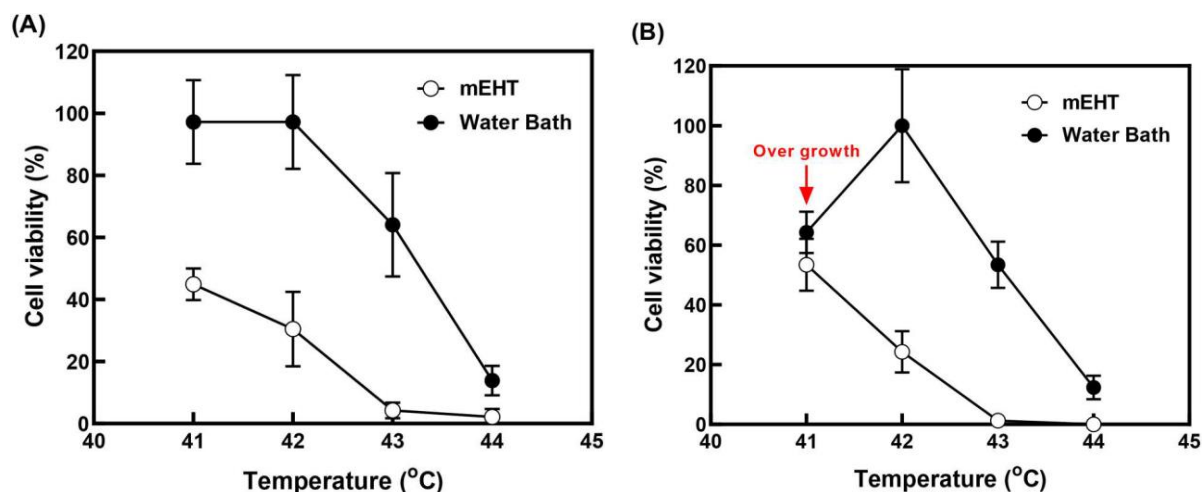


Figure 1. Comparison of the cytotoxic effects of heating with mEHT and that with a water bath. The B16-BL6 cells were heated with mEHT or a water bath for 20 min, and the cellular viability was measured after incubation for 24 h (A) or 72 h (B) at 37°C.

Statistical analyses

All graphs were generated using GraphPad Prism 6.0. Statistical analyses were conducted using one-way analysis of variance (ANOVA), and significant differences between treatments were identified using Bonferroni's post hoc test. All results are shown as mean $\pm$ standard deviation from at least three independent experiments. *, **, and *** indicate significance at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively.

RESULTS

Determination of optimal temperature for EHT

The B16-BL6 tumor cells were treated in suspension with mEHT at 41°C to 44°C for 20 min to determine the optimal temperature for mEHT. The control group was heated at the same temperatures with a water bath. After the treatments, tumor cells were plated on a 96-well plate and incubated for 24 or 72 h, and the viability of the cells was determined by the improved MTT method (Sladowski et al. 1993). We found that mEHT treatment at 41°C for 20 min reduced the viability to 45% to 50% and mEHT at 42°C to 43°C for 20 min reduced the viability to almost 0%. Meanwhile, approximately 50% to 60% of cells were viable after heating at 43°C for 20 min with a water bath (Figure 1(A)). Our result that mEHT treatment significantly reduced cell viability suggests a potential role of mEHT in cell death induction; however, further apoptosis and necrosis assays are required to confirm this effect.

Effect of mEHT treatment time on tumor growth in an animal model

The effects of mEHT in combination with KME treatment (intravenous) on the intradermal B16-BL6 melanoma that is grown in the back of C57/BL6 were investigated. Tumors were treated twice with mEHT on 7 and 11 days after melanoma cell inoculation. The tumor size on day 7 was 0.6–1.6 mm in diameters. The mEHT was done with 1.8–2.4 W RF, which raised the tumor temperature to 43°C, for 10 or 20 min. Tumors were also heated with a water bath at 43°C as the control. The host mice were treated with an intravenous KME injection simultaneously with tumor cell inoculation. The KME treatment was repeated five more times beginning the next day after tumor cell inoculation at 4–

day intervals, in a total of six treatments (Figure 2). We found that the treatment of mEHT alone for 10 min did not show the reduction of tumor size whereas KME alone treatment significantly inhibited the tumor growth. The combinational treatment of mEHT and KME was slightly more effective than KME alone for tumor growth inhibition (Figure 3(A)). However, the treatment of mEHT for 20 min with KEM combination treatment showed the excellent effects in suppressing tumor growth (Figure 3(B, C)). These finding suggest that the co-administration of mEHT and KEM has strong effect on inhibiting tumor growth.

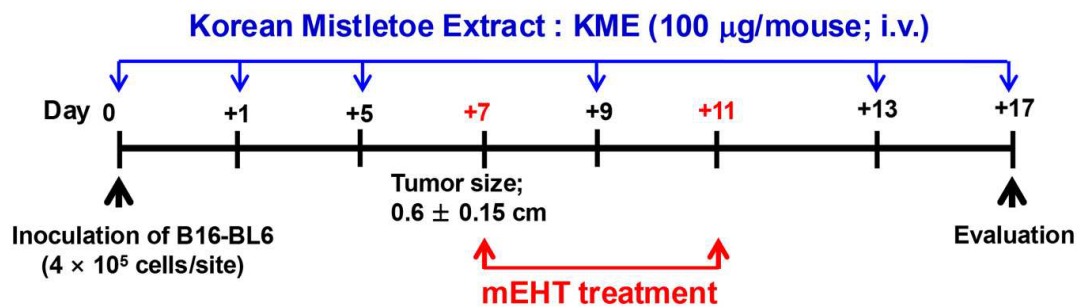


Figure 2. Schedule of mEHT and KME treatment.

Effect of mEHT treatment on the mode of cytokine production by TAMs

Macrophages have two major groups that are classified into M1 and M2 (Mosser and Edwards 2008). M1-type macrophages are activated by LPS or IFN- γ and produce high IL-12 concentrations, which cause inflammatory responses and suppress tumor growth and metastasis. Contrastingly, M2-type macrophages secrete high IL-10 levels and low IL-12 levels and are involved in the anti-inflammatory response, wound healing, and tumor development (Chanmee et al. 2014). Solid tumors are composed of not only malignant cells but also immune cells recruited from the blood circulations (Martinez and Gordon 2014). Among these immune cells in tumor tissue, macrophages are common, and most macrophages are tumor-associated macrophages (TAMs). These TAMs are mainly of the M2 type and actively promote tumor growth (Pollard 2004; Galdiero et al. 2013; Noy and Pollard 2014).

We treated the macrophages with mEHT at 43°C for 20 min and then incubated them at 37°C to investigate the effects of mEHT on the macrophage differentiation pattern. We found no notable changes in the IL-12 levels, which is a typical cytokine that is produced by M1-type macrophages, following mEHT treatment (Figure 4(A)). Meanwhile, the pro-inflammatory cytokine levels, such as TNF- α , were increased by mEHT (Figure 4(B)). IL-10 is a typical cytokine that induces macrophage polarization to M2 type in macrophage differentiation that is affected by tumor cells (Mantovani and Locati 2013). We found no noticeable IL-10 level changes when macrophages were stimulated with viable tumor cells in a heat-treated dead tumor or mEHT-treated tumor cells (Figure 4(C)). Contrastingly, the IL-10 production was significantly lowered when KME was added to the medium, which indicates that KME is capable of effectively inhibiting macrophage differentiation (Figure 4). Overall, these results indicated that mEHT and KME co-treatments inhibited the M2 macrophage differentiation, which are associated with tumor growth and progression.

Combined effect of mEHT and KME treatment on the tumor-specific immune response

Lymphocytes obtained from spleens were co-incubated with B16-BL6 melanoma cells in vitro to investigate whether mEHT can increase the cytotoxic T lymphocytes (CTLs) activity. The CTL activity showed a slight increase compared to the non-treated controls in both groups treated with mEHT or KME alone. However, the combined mEHT and KME treatments were more significantly effective in increasing the cytotoxicity (Figure 5(A)). Cytotoxic T cells act on tumor cells by releasing cytokines, such as IFN- γ , TNF- α , and TNF- β (Schroder et al. 2004). Especially IFN- γ , which is produced by CD4+ T helper cells, enhances the cytotoxic capacity of T lymphocytes on tumor cells (Pardoll and Topalian 1998). Concurrently, cytotoxic T cells produce IFN- γ in the process of killing tumor cells. Thus, we analyzed IFN- γ in the obtained supernatant from the mixed cultures of mouse spleen cells and tumor cells. Figure 5(B) shows that IFN- γ production was the highest in the mEHT + KME treatment group compared to the control and other treatment groups.

We further investigated the alteration of granzyme, which is a serine protease that is released by cytoplasmic granules within cytotoxic T cells. Granzyme-induced apoptotic cell death is one of the main mechanisms underlying the tumor cell elimination by the cytotoxic T cells (Trapani and Smyth 2002). Consistent with IFN- γ production, the combination treatment of mEHT and KME released the greatest amount of granzyme compared with other groups (Figure 5(C)). These results support that mEHT treatment enhances the cytotoxic activity of T cells, resulting in an increased anti-tumor effect by combined therapy with Korean mistletoe extracts.

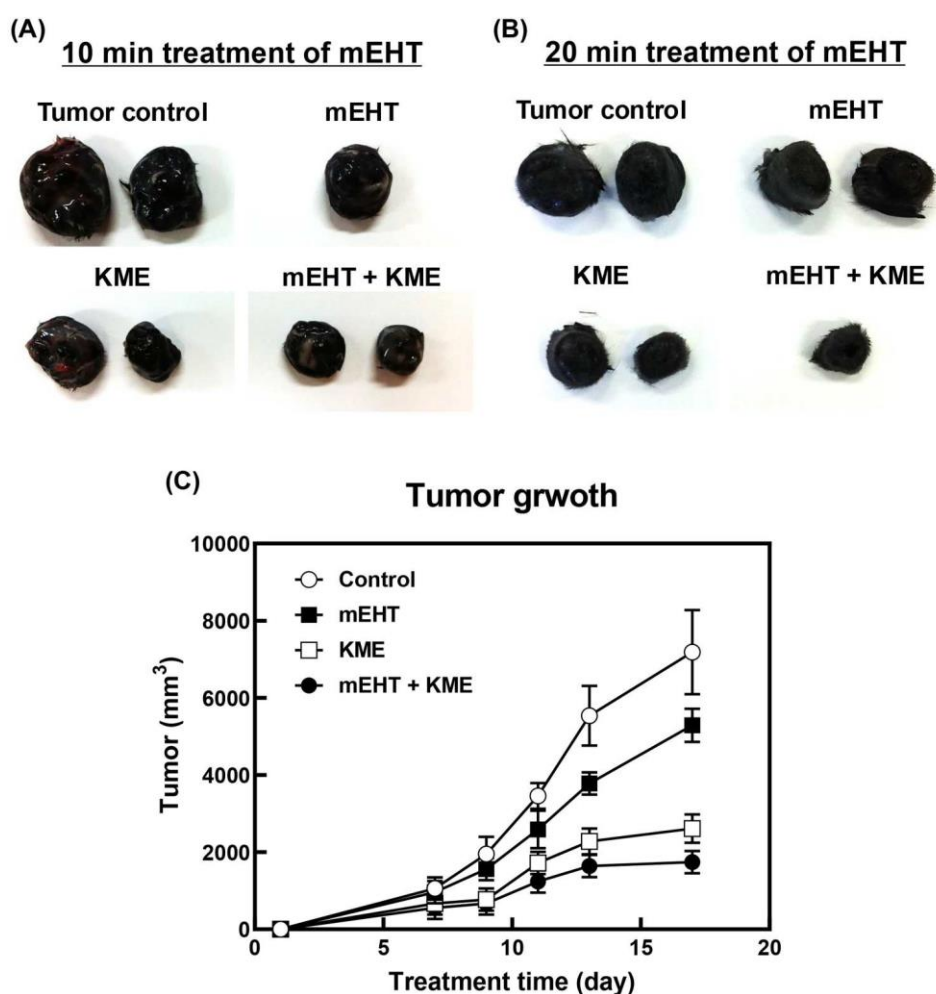


Figure 3. The inhibitory effect of mEHT and KME on tumor growth. B16-BL6 melanomas were obtained from the control (water bath heating), mEHT, KME, and mEHT + KME-treated groups. (A) the mEHT-treated group had 10 min on each indicated day. (B) The mEHT-treated group had 20 min. (C) Tumor size of the control, mEHT, KME, and mEHT + KME-treated B16-BL6 melanomas was measured. Five mice were used in each group.

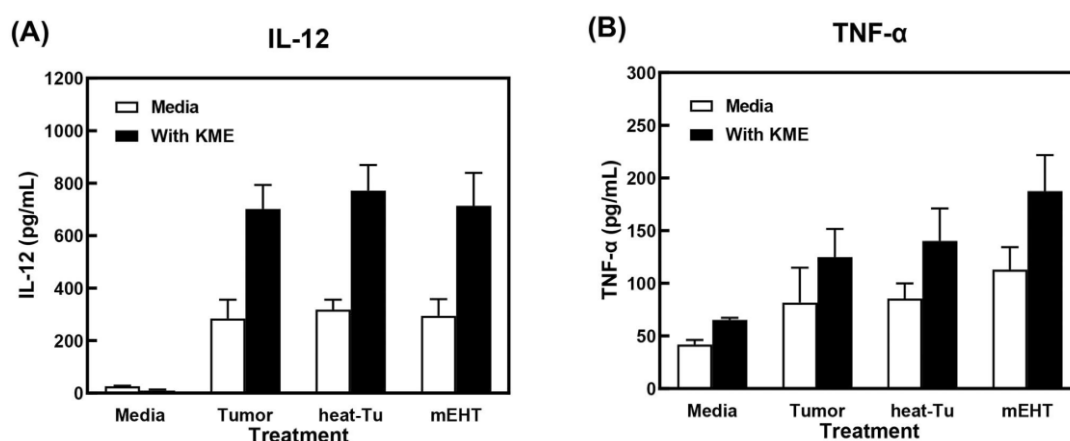
Combined effect of mEHT and KME treatment on antibody production

Tumor antigen-specific antibodies play an important role in anti-cancer immune response because they mediate antibody-dependent cellular cytotoxicities (Scott et al. 2012). Additionally, tumor antigen-specific antibodies bind to tumor cell surface receptors and induce apoptotic tumor cell death (Scott et al. 2012). Therefore, we investigated whether mEHT treatment influences tumor-specific antibody production. Mice were immunized by injecting melanoma tumor cells treated with mEHT alone or in combination with KME.

Three days after injection, the tumor-specific antibody levels were checked in the serum samples of mice using an ELISA. We found that the mEHT treatment alone enhanced the antibody production against melanoma. The combined treatment of mEHT and KME was significantly more effective than mEHT alone in increasing antibody production (Figure 6).

Induction of anti-tumor immune response using the mEHT-treated melanoma as a tumor vaccine

The potentiality of mEHT-treated tumor cells as a tumor vaccine to induce specific anti-tumor immunity in mice was investigated. Mice were immunized with mEHT-treated B16-BL6 melanoma cells. Mice were injected with viable tumor cells 2 weeks after immunization. Then, 2×10^5 tumor cells were subcutaneously injected into the back of mice after 24 h of incubation. The production of IL-2 and IFN- γ in splenocytes, which are typical Th1-type cytokines that are involved in enhanced cellular immune responses (Schroder et al. 2004; O'Garra and Vieira 2007), was then examined after 2 weeks of tumor injection. We observed a significantly increased IL-2 level in the spleen cells of the immunized mice without re-stimulation. Additionally, IL-2 production was further increased when re-stimulated with syngeneic melanoma B16 cells. However, no significant change was found in the IL-2 production in the splenocytes from mice that are re-stimulated with allogenic colon 26-M3.1 carcinoma cells (Figure 7(A)). IFN- γ production was increased when re-stimulated with syngeneic melanoma (Figure 7(B)). Thus, this result suggested that mEHT might enhance the cancer-specific immune response.



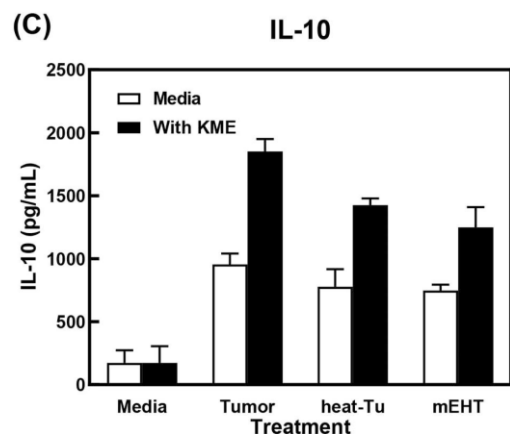


Figure 4. Effect of mEHT treatment on cytokine production by the stimulation of macrophages (A) IL-12, (B) TNF- α , and (C) IL-10.

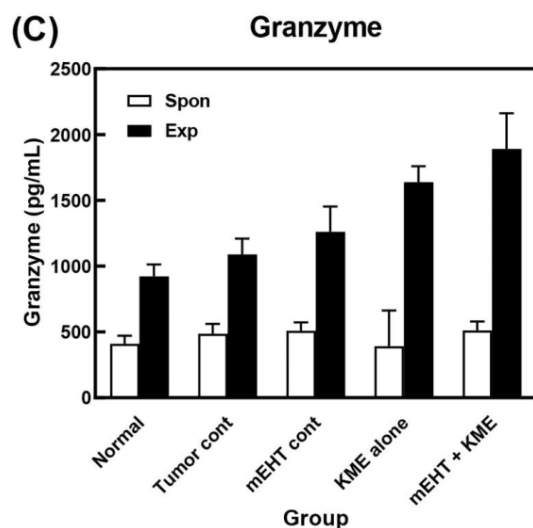
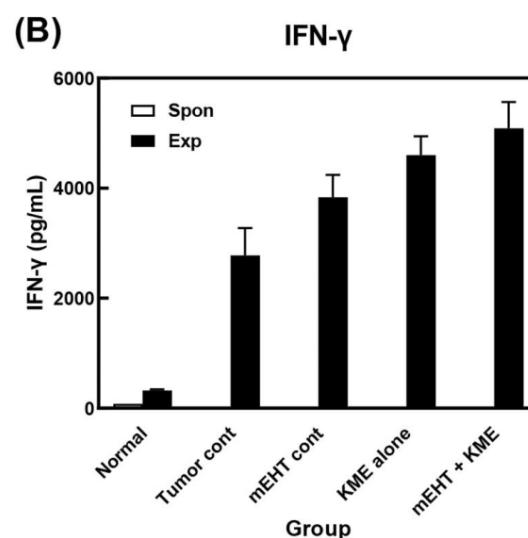
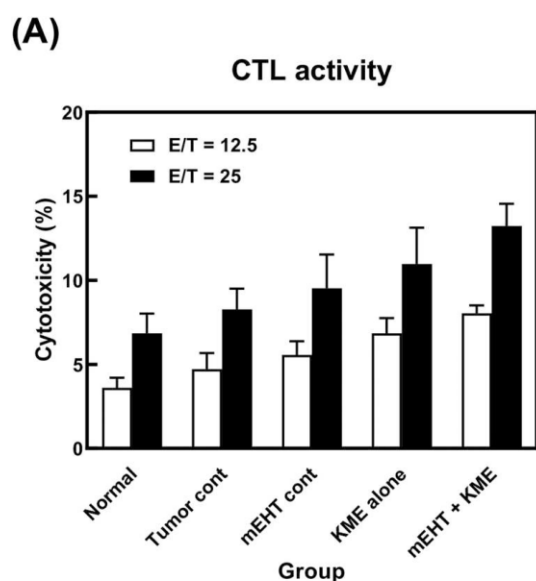


Figure 5. Effects of mEHT and KME treatment alone or in combination on tumor-specific immune responses. (A) T cell cytotoxic activity against B16-BL6 was measured at ratios of effector/target cells (E/T ratio). The IFN- γ (B) and granzyme (C) levels were measured in the obtained supernatant from the mixed cultures of mouse spleen cells and B16-BL6 tumor cells.

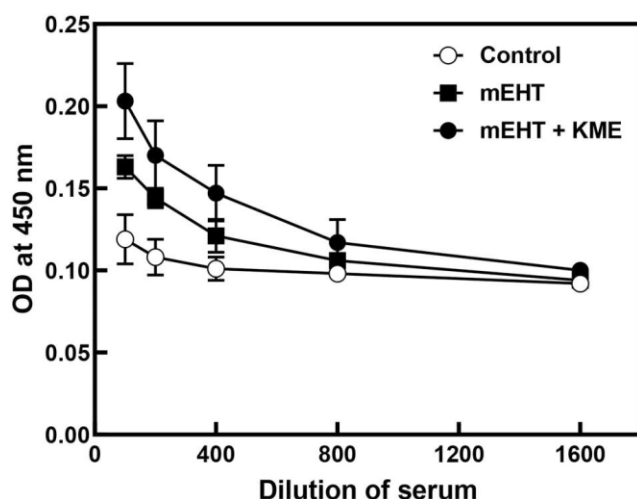


Figure 6. Detection of tumor-specific antibody production in mouse serum samples using ELISA. Mice treated with mEHT alone or mEHT and KME had significantly higher IgG levels.

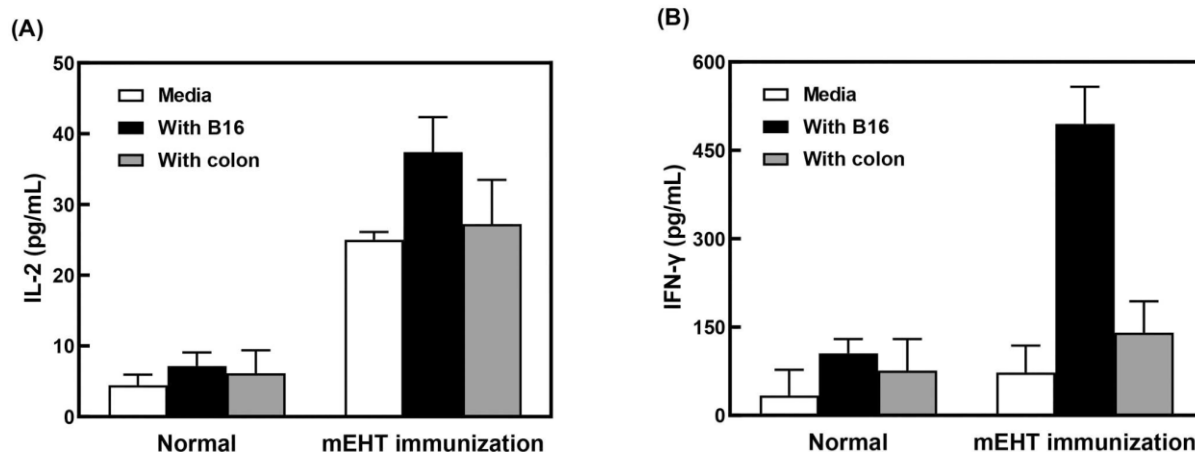


Figure 7. Production of tumor-specific cytokines by immunization with mEHT-treated tumor cells. IL-2 (A) and IFN-γ (B) levels are measured from the splenocytes of normal mice and mice immunized with mEHT-treated tumor cells.

DISCUSSION

EHT is a powerful enhancer of chemotherapy and radiotherapy (Horsman and Overgaard 2007). Additionally, recent reports strongly indicated that mild hyperthermia or fever range whole-body heating is an effective enhancer of the immune responses against infection and cancers (Wang et al. 2001b; Skitzki et al. 2009a; Evans et al. 2015). We have been interested in the possibility that EHT may enhance the anti-cancer effects of various complementary and alternative therapies. To our knowledge, little has been explored about the potential usefulness of combining EHT with alternative anti-cancer therapy with natural compounds that have been known to improve anti-cancer immunity. Mistletoe preparations are used as immuno-stimulating agents in cancer treatment and have been shown to improve the quality of life (QOL) mainly by increasing the immunity in several clinical trials over the years (Bussing et al. 2012; Kim et al. 2012; Troger et al. 2014). Therefore, we hypothesized that a combination of EHT and mistletoe therapy would significantly enhance the immunity of patients with cancer, resulting in an improved QOL.

In recent years, the anti-tumor effects of EHT have become increasingly evident due, at least in part, to the improvement of the immune responses (Frey et al. 2012). TNF- α , which is a pro-inflammatory cytokine, affects the class II MHC expression together with IFN- γ (Trinchieri 2010). Accordingly, the enhanced TNF- α production by mEHT treatment can be concluded to be associated with antigen-presenting T cells after the processing of dead cancer cells by macrophages. In tumor progression, TAMs are generally converted to M2-type macrophages, which are characterized by high IL-10 levels and low tumoricidal activity (Mantovani and Sica 2010; Jeong et al. 2023). Such TAMs have been shown to support a favorable microenvironment for tumor growth and tumor cell survival (Pollard 2004). Therefore, IL-10 secretion inhibition is helpful for suppressing the macrophage differentiation to M2 type (Ahn et al. 2024), resulting in anti-tumor efficacy of mEHT and KME combined treatment (Figure 3). Our data indicate that mEHT and KME combination therapy suppresses IL-10 production and increases TNF- α secretion, suggesting a shift from the M2 immunosuppressive phenotype toward the pro-inflammatory M1 phenotype (Martinez and Gordon 2014). This shift in macrophage polarization enhances tumor immune surveillance and contributes to the observed inhibition of tumor growth.

The combination of mEHT and KME was effective in inhibiting tumor growth and increasing CTL activity (Figure 4). Additionally, the immunization of mice with tumor cells treated with mEHT and KME significantly boosted the antibody production against tumor cells and increased IFN- γ and granzyme secretion. Thus, immunization with mEHT-treated tumor cells mainly contributes to T cell-mediated immunity activation. Importantly, we have observed a significantly increased immune response to tumors after pre-immunization with mEHT-treated tumor cells. The antibody titer against mEHT-treated melanomas, which act as antigens, was also examined (Figure 6). The use of cancer vaccines is based on the expectation that the host immune systems recognize the attenuated tumor cells as an antigen and develop immunity. However, tumor cells are often poorly immunogenic. Thus, developing an adjuvant to increase the immunogenicity of tumor cells is necessary to overcome this drawback. The combination of mEHT and KME treatment for the tumor cells may be used to augment the tumor cell immunogenicity and increase the anti-cancer vaccine effectiveness.

Heated tumor cells generate HSPs, which initiate increased tumor antigen presentation, resulting in NK cell and APC activation (Multhoff 2009a). The induction of tumor cell apoptosis by mEHT increases HSPs release, thereby contributing to the tumor-specific immune response (Tsang et al. 2015b). Therefore, examining the effect of combined therapy on HSP production would be interesting. Additionally, detailed studies about the synergistic effect of combined treatment on NK cell activation are intriguing because both EHT and mistletoe extract show the NK cell enhancement function (Yoon et al. 1998a; Multhoff 2009b).

In summary, we have found that the combinational treatment of mEHT and KME improved the anti-cancer activity and immunological response in mice, which suggests the potential of mistletoe preparation as a partner of EHT for cancer therapy. Our findings suggest that this combination therapy may exert its effects through multiple mechanisms, including enhanced antigen presentation, modulation of the tumor microenvironment, increased tumor-specific antibody production, and induction of immunogenic cell death (Kim et al. 2024). Future studies should focus on elucidating the precise molecular pathways underlying these effects, as well as investigating the potential application of mEHT-treated tumor cells as a cancer vaccine. Thus, our findings

contribute to a deeper understanding of how mEHT and KME synergistically enhance antitumor immunity and pave the way for novel therapeutic approaches in cancer treatment.

Disclosure statement

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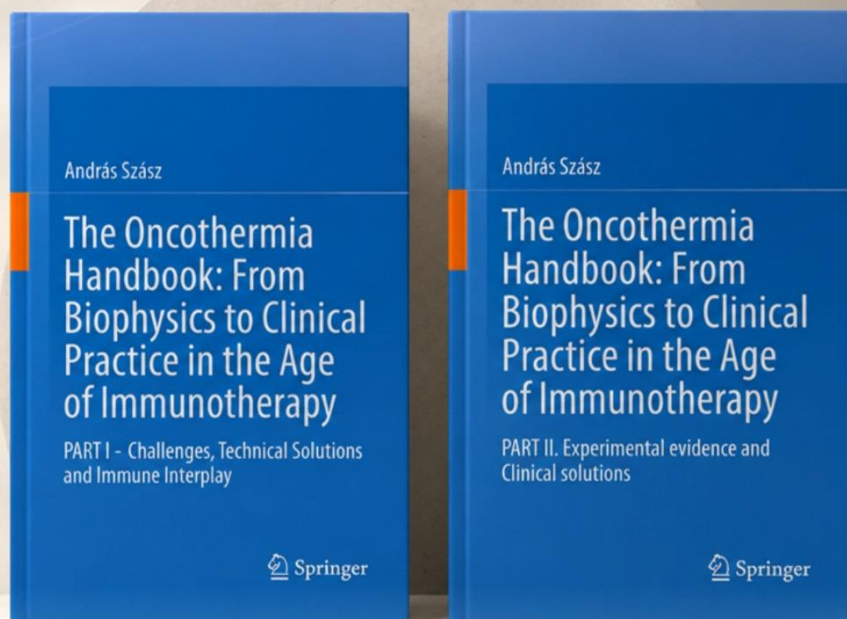
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