The Immunogenic Connection of Thermal and Nonthermal Molecular Effects in Modulated Electro-Hyperthermia

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Abstract

Hyperthermia in oncology is an emerging complementary therapy. The clinical results depend on multiple conditional factors, like the type of cancer, the stage, the applied treatment device, and the complementary conventional therapy. The molecular effect could also be different depending on the temperature, heating dose, kind of energy transfer, and timing sequences compared to the concomitant treatment. This article examines the molecular impacts of a specific technique used in oncological hyperthermia called modulated electro-hyperthermia (mEHT). What sets mEHT apart is its emphasis on harnessing the combined effects of thermal and nonthermal factors. Nonthermal energy absorption occurs through the excitation of molecules, while the thermal component ensures the ideal conditions for this process. The applied radiofrequency current selects the malignant cells, and the modulation drives the nonthermal effects to immunogenic cell death, helping to develop tumor-specific antitumoral immune reactions. The synergy of the thermal and nonthermal components excites the lipid-assembled clusters of transmembrane proteins (membrane rafts) as the channels of transient receptor potentials (TRPs), the heat-shock proteins (HSPs), the voltage-gated channels, and the voltage-sensitive phosphatases (VSPs). All these transmembrane compartments channeling various ionic species (like calcium and proton) interact with the cytoskeleton and are involved in the apoptotic signal pathways.

Keywords

Thermal, Nonthermal, Membrane Rafts, TRP, VSP, HSP, Cytoskeleton, Polarization, mEHT, Immune Effects, Abscopal Effect

1. Introduction

Electromagnetism appears as a continuous challenge in biology. The search for the use of electromagnetic effects for therapies ignited considerable research and hypotheses [1]. The ionizing radiation beam (high energy electromagnetic spectrum) shows immediate effects. The absorbed energy spectacularly destroys the biomaterial in the path of the beam. The non-ionizing radiation has less energy and more complexity. It modifies the chemical compounds and reactions and could impact enzymatic processes [2]. The complexity of living structures with physiological self-regulations challenges the therapeutic applications of non-ionizing effects. The challenges highlight the physiological importance of electric currents, deriving much intensive research, including neuroscience [3] and controlling the cellular effects [4]. A general hypothesis of "biologically closed electric circuits" (BCEC) introduced bioelectromagnetic homeostasis based on the existence of intrinsic electric currents in the body [5] [6], modified by malignant diseases [7] [8]. The pathological disorders [9] and wounds induce intrinsic injury currents [10], driven by the automatic biological charge transfers induced by the tissue- repair process [11] [12]. Observations have been made regarding the biological effects of low-level, non-stationary magnetic fields [13] [14]. The bioelectromagnetic effects may have resonance characters [15] [16] [17].

All electromagnetic interactions deliver energy to the biomaterials. The energy could be realized by heat (which may increase the temperature) and electron excitation (which makes chemical changes). These effects are naturally combined. The bioelectromagnetic interactions partly modify the chemical bonds and structure of compounds with electromagnetic forces, while the part of the energy absorption heats the target. The preferences may change the treatments. For example, radiotherapy breaks the DNA strands, modifying the chemical bonds, where the heating is an adverse effect, while the focus of hyperthermia is to heat and neglects the direct chemical effects of the electric field. Initially, hyperthermia used both the field and heat effects combined in the middle of the 18th century, but later it split by dominant electric (by French doctor Arsene d'Arsonval) and heat (by Danish doctor Kristian Overgard) effects. To produce, control, and understand the heat effects were more accessible, promoting its worldwide spread and helped by some industrial devices manufactured by Siemens in the early 20th century.

Nowadays, a novel approach tries again to combine the thermal and nonthermal factors of non-ionizing radiation using modulated radiofrequency (RF) signals [18]. The method (modulated electro-hyperthermia, mEHT [19]) applies definite heating in the fever range [20] and bioelectromagnetic effects in the bioprocesses using the nonthermal electromagnetic activity [21] [22] in the energy range selectively exciting transmembrane proteins on the malignant cells [23]. The principal selectivity of mEHT concentrates on the physiologic specialties of malignant cells and how they differ thermally and electrically from healthy ones. The malignant cells have a higher metabolic rate that drives the RF current by high ionic density in the tumor-cell microenvironment (TME). Moreover, healthy cells maintain homeostatic electrolyte concentrations in various regions by wellcontrolled electrolyte balance having body electrolytes in the right concentrations regulated by heart, kidney, and neurological function, controlling the acid- base, fluid concentration balance, oxygen delivery, carbon dioxide transport, and other processes in the complex human body. The kidneys maintain a massive sodium regulation, which balances the important Na/K balance and calcium concentration for cellular functions. Calcium is involved in the function of enzymes and serves in signal transduction pathways, acting as a second messenger, in neurotransmitter release from neurons, in contraction of all muscle cell types, and in fertilization. Cancer cells alter the electrolyte balance concentrations. Some tumors have hypercalcemia, and the dysregulated pH causes electrolyte imbalance in cancer.

The measured impedance between healthy and cancerous tissues exhibits significant differences [24]. This impedance assists in selecting appropriate radiofrequency (RF) parameters [25] and enhances current density within the cancerous tissue [26] [27]. MRI images the selection showing high RF current density in the tumor [28] and prove the self-selection of the malignant region by the current flow [29] [30]. Electrical impedance tomography pro provides further feasibility of focusing on impedance differences [31]. The preclinical experiments in various investigations show the temperature differences between the tumor and its surroundings (Figure 1).

The growing temperature on the membrane makes a particular thermal impact compared to the conventional heating (water-bath, wHT) (Figure 2).

The mEHT focuses on the complex equilibrium of the human body [39] with an appropriate technical solution [40], synergizing thermal and nonthermal energy components [41] by strong interaction of heat production with field effects [42]. The central concept of mEHT uses the natural homeostatic control of the human body [43], using a low-frequency modulation to stimulate healthy homeostatic regulation [44] [45].

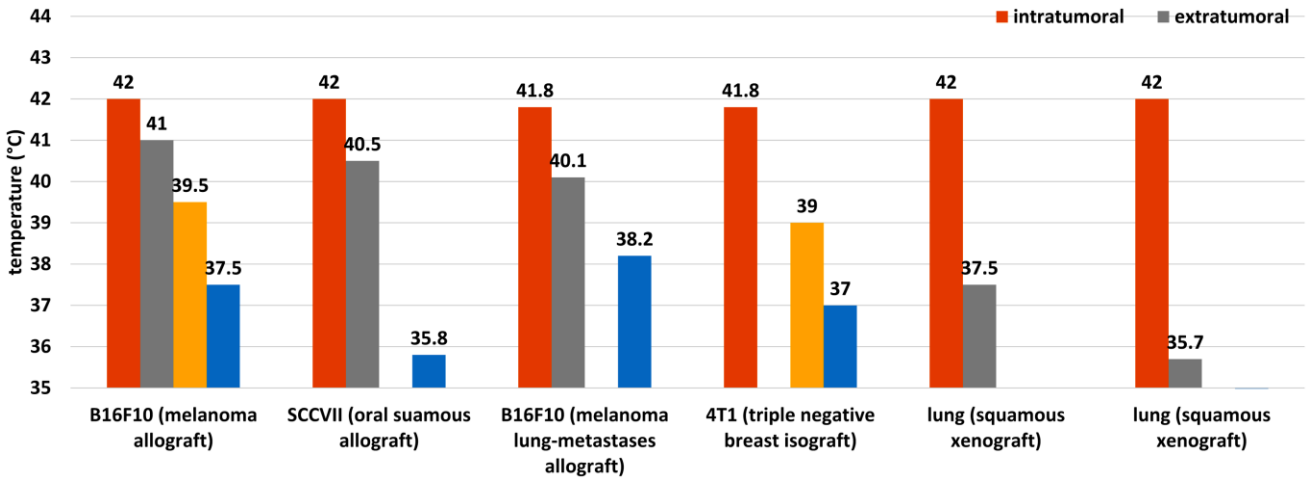


Figure 1. Temperature differences between the tumor and its surroundings, B16F10 (melanoma allograft) [32], SCCVII (oral squamous allograft) [33], B16F10 (melanoma lung-metastases allograft) [34], 4T1 (triple negative breast isograft) [35], lung (squamous xenograft) [36], lung (squamous xenograft) [37].

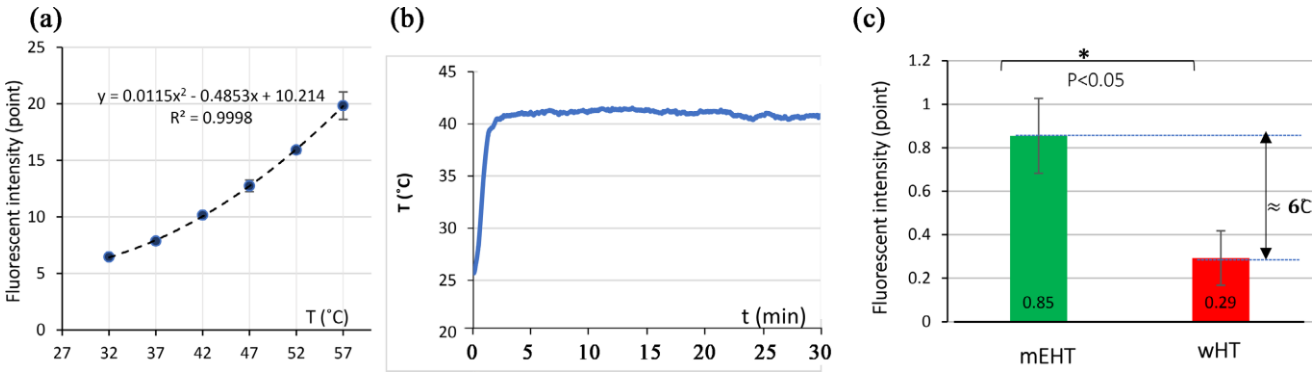


Figure 2. Measurement of the membrane temperature [38]. (a) Calibration of the membrane temperature by 10 μM DIL dye (RPMI + 10% FCS + 1% L-glutamine + 0.4% gentamycin). HT29 (human CRC cell line). Dilutions were kept at discrete temperatures for 30 minutes. (b) The temperature of the medium of the cell culture. (c) Significantly higher membrane temperature was achieved with mEHT than with wHT.

2. Thermal Impact

The intensive metabolic activity of the malignant cells [46] increases electric conductivity by the ionic density in the TME. Furthermore, the tumor has a higher water content [47], which further increases the electric conductivity of the tumor. In this way, the entire tumor conducts better than its neighbor [48] [49] [50] [51]. An additional selection factor is that the malignant

processes destroy the networking orders [52] [53] [54]. The presence of the disorder leads to an increase in the dielectric permittivity (ε) of the microregion [55] [56] [57] [58]. Consequently, the electric current will naturally follow the most accessible route, which is typically the most conductive path, thereby flowing through the tumor. The water content within the tumor microenvironment (TME) interacts with the membrane [59], forming various bonds [60] and significantly impacting the membrane's functionality. This phenomenon results in a low specific absorption rate (SAR) but a high voltage drop [61], facilitating the excitation of raft proteins [62] by the signal. The electrostatic charge of the membrane attracts ions from the extracellular matrix (ECM), producing a diverse effect that is sufficient to establish a transmembrane potential [63].

Selective raft heating makes a higher cell-killing rate with apoptotic processes than conventional homogeneous water bath heating (wHT). A calibration curve by wHT describes the apoptotic rate by temperature. The mEHT heterogeneous heating has a higher impact on the membrane proteins than wHT (Figure 3).

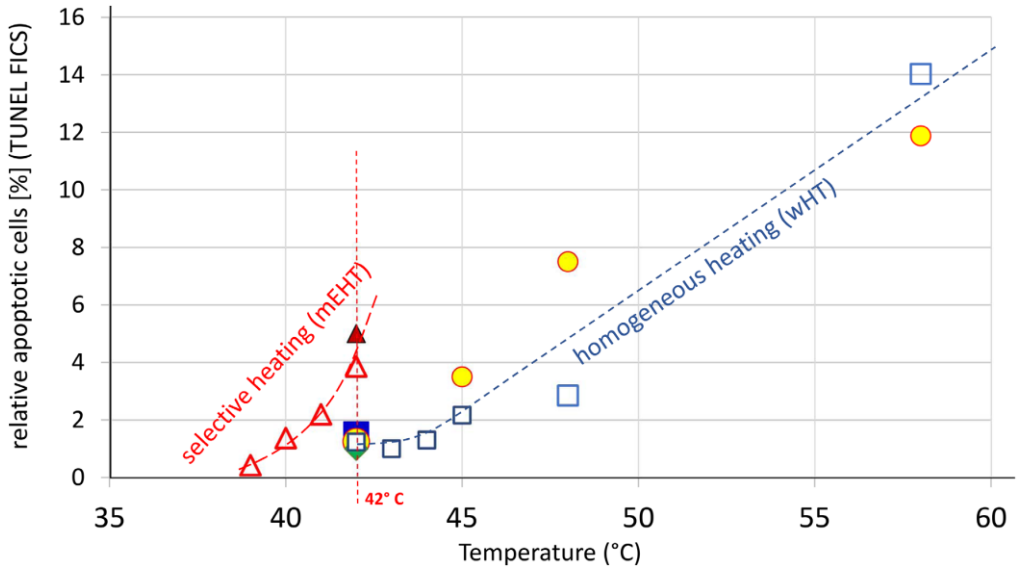


Figure 3. Thermal calibration with wHT measured with cell-lines U937 (□) [64], HepG2 (○) [65], CT26 (◆) [66], and the mEHT with U937 (△) [64], and HepG2 (▲) [65], cell-lines. RF homogeneous heating with a conventional capacitive device for HepG2 cells is also given (■) [65].

The transient receptor potential (TRP) channels are a set of transmembrane proteins and form a family of cation control channels [67]. These channels rectify the ionic transport, mainly calcium Ca2+, through the membrane. The rectification parameters primarily depend on thermal conditions sensing the relative to homeostasis oppositely in hot and cold temperatures. The hot sensing shifts the Ca2+ ion-flux to the opening direction [68], while the cold one shifts oppositely [69] to the closing side. TRPs regulate the membrane polarization, function as primary thermal sensors of cells [70], inducing action potential for physiological sensation, and cover chemo-, mechano-, and photosensation [71] of individual cells. The intracellular organelles and cellular compartments also have TRP channels in various vesicular processes [72] [73]. The Ca2+ intra and extracellular transmembrane ionic exchanges have a decisional role [74], allowing the individual cells to react to all intra and extracellular stimuli

signals. The intracellular TRPs actively participate in membrane fusion and fission, signal transduction, and general vesicular homeostasis [73]. The TRPV5 and TRPV6 are the only TRPV channels that are highly selective for Ca2+ [75]. Others have low or no selection on this ion. The TRPV1 is also a proton channel [76], which lowers the pH of the cytosol [76]. However, the Ca2+ -selective ORAI channel [77] has tight interactions with non and weak Ca2+ selective TRP channels and may activate the TRPCs while that may localize the ORAI [78].

The TRP channels have an exceptionally high temperature-coefficient Q10 [79]. It is notable that both the enthalpy and entropy components of its transition through the ion-permeability barrier are high [79].

The vanilloid receptors (TRPVs) cover the temperature sensing in mammals from low skin temperature (~25˚C - 45˚C TRPV4) to the necrotic high up to ~50˚C - 60˚C, TRPV2 [80] [81]. The hyperthermia fits TRPV3 (~24˚C - 34˚C) and TRPV1 (~41˚C - 50˚C) ranges [81].

The cell-membrane rafts became in focus [82] and well-studied [83]. The TRP receptors could also be a part of these clustered microdomains in the membrane and present effective thermosensors of the cell [84]. There are essential observations indicating a coherent cluster structure of a large number (~10^5) of voltage- gated ionic channels [85] [86], and it could have transient receptor potential (TRP) receptors in one temperature-sensing domain [84]. Small temperature changes may affect the TRP channels with membrane lipid assistance in the raft microdomain. The membranes are inhomogeneous, which is enhanced by the mild temperature change. Notably, the activated TRPV1 channel's ionic current may disappear at the multiple repetitions of the thermal ignition [87]. The opening of the TRPV channel for ionic current needs a relatively large enthalpy, while its closing depends less on the provided energy [88]. This asymmetry works oppositely in cold-activated channels like TRPM8 [89].

The primary energy absorbers in the mEHT method are the cytoplasmic membrane rafts, heating them selectively. The selection is based on these microdomains' high specific absorption rate (SAR). The thermal influence of mEHT has traditional hyperthermic functions, promoting the cell death in various ways [90]. Nevertheless, the difference is significant: the mEHT selectively heats the tumor. The selection focuses on membrane rafts, the cholesterol-stabilized microdomain cluster of transmembrane proteins [82], participating in the membrane dynamics [83]. The rafts have exceptionally high energy absorption from the RF current [91], allowing cellular selection of malignant cells with significantly high raft density [92] without substantially heating the healthy ones [93]. The concentrated heating of the molecular groups in the membrane rafts creates a heterogenic situation, where the rafts heat the entire cell and, in a second step, the tumor [94]. While the tumor, on average, remains in the <40˚C fever range, the rafts reach < 3˚C higher temperatures [95].

The temperature increase of the nano parts in the target is negligible with homogeneous heating [96]. However, when the heating is heterogenic (which is the case of all nanoparticle heating and in the mEHT too), the local SAR could be extremely high due to the small particle size. This is used in nanoparticle heating when the SAR on the nanoparticles could be as much as more than 1 MW/g (1,000,000,000 W/kg), depending on the absorber's concentration [97].

The inhomogeneous electric field in the case of mEHT, where the dielectrophoretic force drifts the rafts forwards, gives an additional factor to increase the micro-heterogeneity of cellular heating (Figure 4). This process exhibits a significant level of selectivity because the dielectric permittivity of the transmembrane proteins is at least two orders of magnitude higher than the permittivity of the surrounding membrane through which they traverse [98].

The TRP regulative processes are dynamic. The transmembrane protein displaces by the temperature action [99]. In malignant cells, the motility of transient receptor potential (TRP) channels is more pronounced compared to their healthy counterparts [100]. As a result, the drift movement of these channels indicates regions of higher energy density, where the specific energy absorption (SAR) is also elevated. The SAR values increase specifically at these points of the membrane known as micro-contacts (Figure 5(a)).

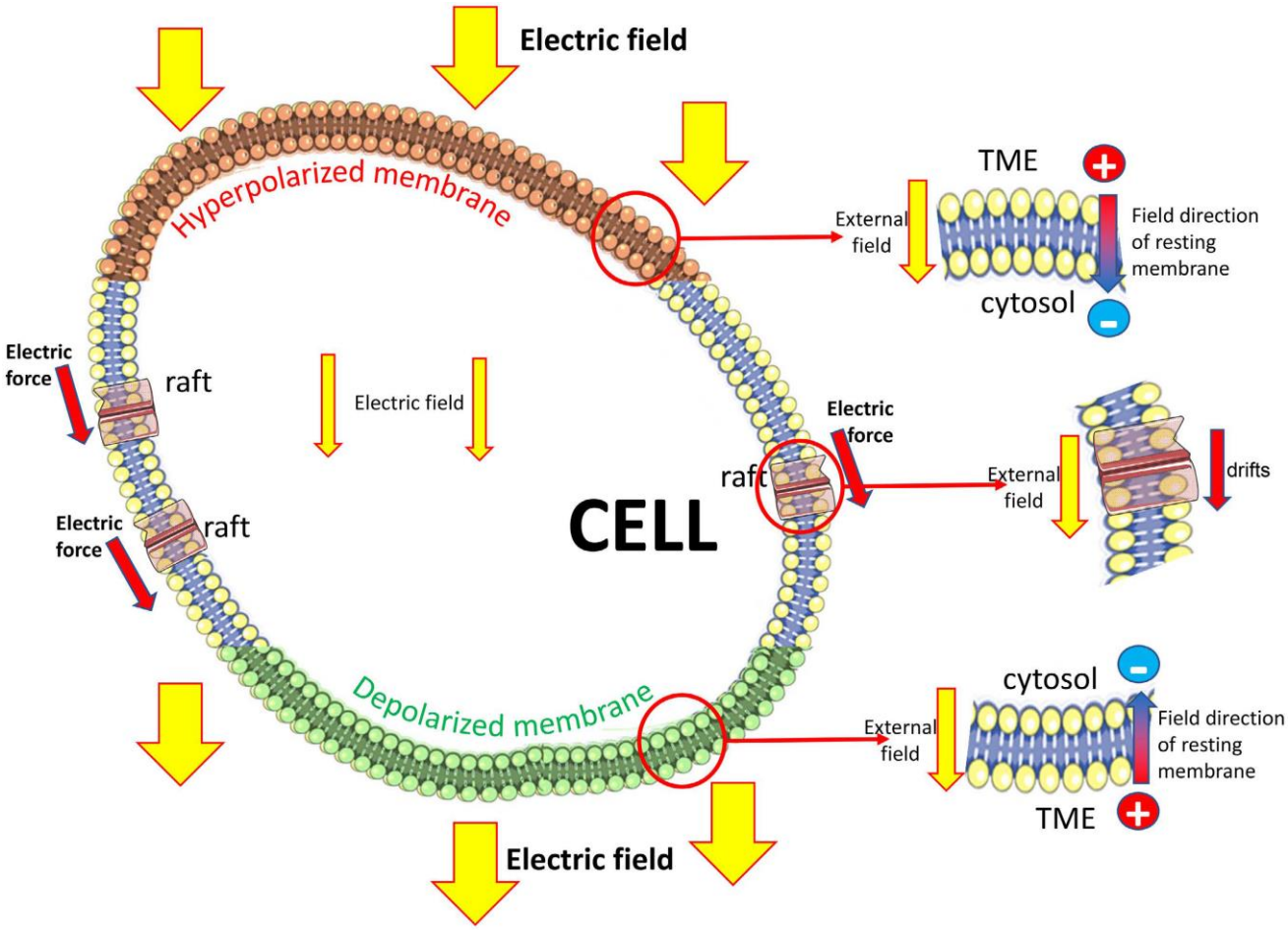


Figure 4. The electric field polarizes the membrane. Hyperpolarizing happens on one and depolarizing on the opposite side of the cell. The rafts have the electrophoretic force to drift by the electric force. The mEHT uses a 13.56 MHz carrier frequency. Consequently, the direction of all processes changes by ~0.07 μs, and so the movable proteins will be enriched in both sides of the cell.

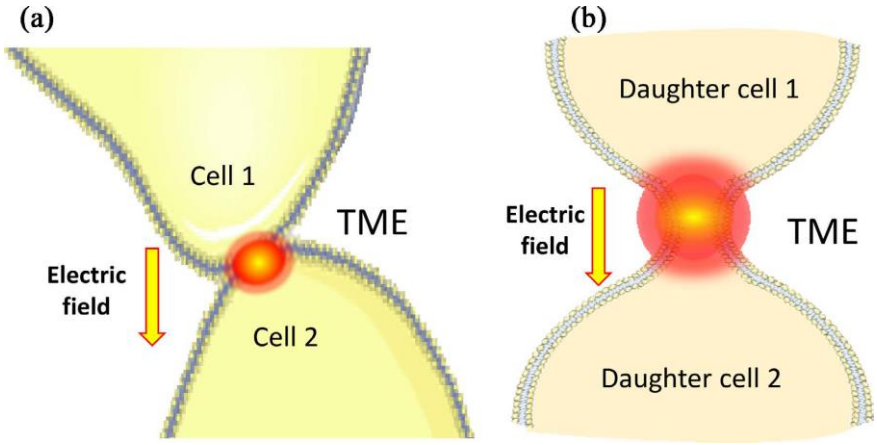


Figure 5. Developing extra hot spots on the cell membrane. (a) The cells touch each other, which enhances the SAR value and increases the spot's temperature. (b) The cytokinetic phase of mitosis has a "neck" between the forming daughter cells. This small area behaves like a touching point shown in Figure 5(a).

The telophase → cytokinesis phase forms another significant vital direct contact during the mitotic spindle, which has high importance in the proliferative malignant cells. The neck between the just-forming daughter cells induces cataphoretic forces [101]. The small cross-section of the neck may absorb an extremely high SAR when its directional position matches to electric-field vector. The absorbed energy by the cytokinetic "neck" depends on the cellular orientation, having a maximum when the field lines are directly parallel to the cytokinetic "neck" [102] [103]. The exceptionally concentrated energy may arrest the cytokinesis and block malignant proliferation [104] (Figure 5(b)).

In the outer membrane, dominantly the TRPV1, while in the membrane of intracellular compartments, the TRPV3 is thermally activated by mEHT's heterogeneous heating. The TRPs actively participate in membrane fusion and fission, signal transduction, and general vesicular homeostasis [75].

The thermal load has another well-known consequence: the development of protective chaperoning heat-shock proteins (HSPs) [105] [106]. The HSPs are also part of the complex regulation of the living organization, resulting in cellular defense or promoting cell death [107]. The complexity of HSPs questions their role as a "friend" or "foe" [108] [109] [110]. This dual behavior [111] [112] appears to decide their function as inflammatory or anti-inflammatory, pro-tumor or antitumor, immune-stimulatory or immune-suppressant, etc. The intensive thermal stress secrets membrane [113] and extracellular HSPs [114], which may reverse their cell-protecting activity [115]. The HSP expression can link to the plasma membrane processes by mild heat [116], which may cause non-specific clustering [117] by fever-like temperatures, where TRPs are particularly sensitive.

3. Nonthermal Impact

The electromagnetic effects differ between healthy and cancerous tissues. The essential differences appear in the conductivity and dielectric properties of the tissues. The breaking

healthy cellular network in cancer better conducts the radiofrequency current, and its dielectric permittivity (polarizability) is also significantly higher. Further differences appear in the electromagnetic excitability of the signal pathways in the cells due to the expressive contrasts of the healthy and malignant cellular membranes. The low membrane potential and high number of intercellularly unconnected transmembrane proteins appear in malignancy which interacts profoundly differently with the external electric field than the healthy cell. Using the apoptotic calibration in Figure 3, the impact of mEHT for cell-killing is rather significant (Figure 6). The basic structural disruption of healthy order makes the tumor also distinguishable by its electromagnetic interactions.

Any other than thermal stress that influences the homeostatic equilibrium also activates the HSP synthesis [118], which induces the cellular chaperoning function with HSPs. Living objects have not only thermal interactions. The dominant number of living regulation effects is not feasible with thermal effects. Enzymatic reactions and other molecular changes are mostly nonthermal, and their functions are mandatory for life. Thermal conditions are responsible for optimizing the nonthermal chemical reactions, and many biological processes synergize thermal and nonthermal components [119]. RF radiation induces nonthermal effects [120] together with the well-known thermal one for conventional hyperthermia treatments [121]. There is a large family devoted to voltage- gating ionic control [122] [123], which has an also large subfamily of voltage- gated calcium channels [124].

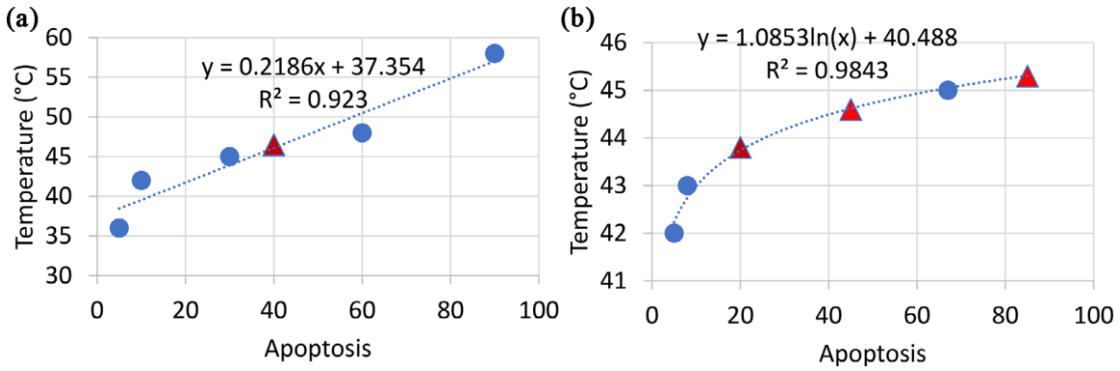


Figure 6. The heterogenic impact of mEHT ( ) compared to homogeneous heating ( ). The percentage of apoptosis shows much higher temperature of the process than the measured 42°C in the cell culture medium. (a) HepG2 [65], (b) U937 [64].

Even the thermally sensitive TRP channels also have nonthermal voltage dependence [125], which is effective in different temperature ranges. The high value of the transition entropy of TRPVs [126] shows that the transition follows Eyring's theorem [127]. This transition explains how the enzymatic reactions decrease the energy barrier with quantum-mechanical effects (e.g., tunneling [128]) and what mechanism is behind the reaction rate changes of catalyzes. The interdisciplinary applications [129] could use the quantum-mechanical considerations [130] [131] of the transition process, making possible a first-order phase transition when only the entropy changes the overall reaction rate, and the temperature remains constant when a new phase appears. The process is nonthermal, at least in its part. In this way, the large entropy in the TRPV transition allows the nonthermal transition when the temperature is unchanged but optimizes the complete process. The high entropy points the structural changes, which have a critical role in the nonthermal activity [132]. The probability of changes has a similar

expression for temperature and electric field [133], which further supports the nonthermal activity. Notable, while the repetition of thermal stimuli decreases the signal level [90], the repetition of the nonthermal chemical stimuli increases it [68].

The electric field produces such excitations, which is impossible with thermal conditions [21] [22]. A notable example showing the exceptional excitation ability of electromagnetic field is that using a broad-band (0.2 - 20 MHz) signal increases the HSP70 expression [134] in such volume that to produce the same rise of HSP70 by temperature, the perturbation should have been 14 orders of magnitude greater [135]. The electric field significantly modifies the cells [136], manipulates protein expressions, and induces extrinsic molecular pathways [137]. The modulated electric field's vibrational effect, along with the associated electro- osmotic process, operates within the intracellular environment [138], potentially triggering various processes within intracellular compartments. The polarization and polymerization may affect the cytoskeleton structure and the pathways that use it. The network of cytoskeleton forms and changes by the various signal transmissions in the cells. These polarizable parts of the cytoskeleton (their main components are the microfilaments, intermediate filaments, and microtubules, and their building blocks the tubulin dimers the actin subunits, and the fibrous subunits) are all interacting with external fields and are all capable of rapid growth or disassembly. The field-sensitive polymerization processes are the most basic of the mitotic rebuilding of the cell-structure of the newly born daughter cells. The membrane rafts incorporating thermosensitive TRPVs could considerably increase the Ca^{2+} influx to the cytoplasm by heating. The Ca^{2+} ionic balance has an important controlling function in homeostasis. It controls several processes in tumorigenesis [139]. It participates in gene transcription [140], cellular motility [141], invasion [142], cell-cycle regulation [143], and angiogenesis [144]. The balance between cell proliferation and apoptosis is tightly regulated by the influx of calcium ions Ca^{2+}. The intracellular concentration of Ca^{2+} plays a crucial role in determining whether a cell undergoes division or apoptosis [145]. Several steps of the killing of cancer cells are Ca^{2+} dependent [146]. The cytosolic and mitochondrial Ca^{2+} overload strongly stimulates the apoptotic processes [147] [148], but, as is usual in complex processes, the low nM Ca^{2+} concentration may help the survival of malignant cells by promoting proliferation by lowering the membrane potential [149] [150] and increasing the malignant differentiation [151]. On the contrary, the rise of intracellular Ca2+ concentrations to μM may support apoptosis [152].

The regulation of various pathways by Ca^{2+}together with its concentration dependence, a temporal component has an important role in tumorigenesis [153]. The amplitude and duration of Ca^{2+}signals involved are different proinflammatory activation of B lymphocytes [154] by decoding its information of amplitude and duration. Amplitude modulation of the Ca^{2+}signal may produce positive or negative antigen response in gene activation of B cells [155] [156].

Bioelectromagnetic interactions make numerous molecular excitations and chemical changes, which thermal interactions cannot achieve [157]. For example, the various intensities of electromagnetic signals make an entirely different activity of the hormone 5-hydroxytryptamine, which could even cause transepithelial potential oscillation [157].

These nonthermal effects characterize the mEHT method [21] [22] and appear in all molecular changes made by thermal effects, even in the TRPV thermal sensors [158] and HSPs [159] [160].

The mEHT uses the gaining possibility of the cell membrane. According to in-silico models, the electric field-strength gain, which refers to the ratio of the induced field within a material compared to the externally applied field, is highest in the cell membrane [161]. Specifically, for frequencies up to a few tens of MHz, the gain remains approximately ≈ 5 × 103 and follows a power-law decrease of 1/f as the frequency increases [161]. Inmore realistic tissue models, the membrane gain varies depending on the cell's position within the tissue, but it never falls below 10^2 in tissue arrangements [162]. In the case of cancer cells, while the intracellular gain is comparable to that of non-cancerous cells, the membrane gain in malignant cells is twice as high as that of their healthy counterparts [162].

The TRPV investigation of activating and deactivating potentials proves their voltage dependence [163] and that thermal conditions optimize voltage action [164]. In addition to heat and electric effects (like potential change or proton influence), the chemical effects (like capsaicin) and proinflammatory cytokines may activate the TRPV1 [165], which is upregulated in many cancer types [166]. The multiple influences make synergy [130] [167]. The multi-sensing behavior makes these channels critical for communication with the changes in TME. The voltage alone cannot wholly activate the TRPV1 channel [168]; the thermal component works for it in synergy, in the complexity of the membrane potential, ligand binding, mechanical force, and temperature [169].

An essential aspect of TRPs activity is its interplay with the cytoskeleton [103], which is based on electromagnetic interactions [170]. The role of the cytoskeleton in signal transduction and its connective role between the intra and extracellular information exchange makes it especially important. The connecting structure of TRP and cytoskeleton allows Ca2+ independent signaling [171]. TRPV1 essentially regulates the dynamics of the cytoskeleton by colocalization and stable binding with microtubules when there it is resting. However, in an excited state, TRPV1 rapidly disassembles the microtubule polymers [169].

The microtubules of the cytoskeletal network have a polymer structure [172]. The loss of the polymerization order of the cytoskeleton probably causes the high motility of cancer cells [173] because it makes the cells especially soft and detachable [174]. The increasing motility induces high metastatic potential and high deformability [175]. The extracellular matrix (ECM) plays a role in the cellular motility of cancer cells connected to its rigidity [176]. The heightened motility observed in cells is likely attributed to the loss of polymerization order within the cytoskeleton [172], resulting in increased cell softness and mobility [173].

The polymerization process follows a chain polymerization model known as Einstein's polymer [177] [178]. However, this model is unable to account for multi-bonding processes where chemical bonds can form branches in tubulins, leading to the creation of various space-filling structures. The reorganization of the cytoskeleton also promotes the formation of multi-strand cases. Multi-strand structures have longer chains compared to single-strand structures due to their multiple free ends and energy centers, making them energetically less favorable. As a result, according to Boltzmann statistics, the concentration of multi-strand chains is lower than that of single-strand chains. There is a relationship between the polymer concentration [M_n] and the polymer length, expressed in terms of the number of monomers, denoted as "n" as

$$[M_n] \propto e^{-\frac{n}{n_0}}$$

where n_0 is constant. Consequently, the high concentration has shorter polymers.

The modulation employed in modulated electro-hyperthermia (mEHT) amplifies the effective electric field, thereby supporting the polymerization and reorganization of the cytoskeletal network. When using 1/f fractal noise modulation [15] [44] [45], this effect becomes more potent due to the continuous spectrum of frequencies present in the non-discrete noise signal. The application of noise modulation bears a resemblance to the harmonizing method [179], which is gaining recognition in the field of physiology [180]. In cancer cells, cytoskeletal polymerization holds particular significance, as the destabilized and incomplete polymerization of the cytoskeleton contributes to increased cell motility and facilitates metastatic spread.

The influence of the modulated electromagnetic field on the cytoskeleton can also involve voltage-sensitive phosphatase (VSP) [181]. Field-controlled phosphorous hydrolysis mediated by VSP could play crucial roles in cytoskeletal restructuring and exhibit resonant-type behavior. VSP, a macromolecule with a voltage sensor and cytoplasmic phosphatase domains [182], regulates the influx of calcium ions Ca^{2+} into cells [183]. VSP is sensitive to external fields and operates within the cytoplasm, allowing the transmission of external field effects to the cell interior. This process generates biochemical signals that may contribute to intracellular organization. Through these signals, it is possible to generate biochemical cues within the cytosol that can control internal processes, most likely including cytoskeletal polymerization. The fundamental mechanism involves membrane depolarization leading to phosphoinositol hydrolysis [184]. This is a reversible decomposition reaction that the external electric field may modify.

Phosphorylation plays a crucial role in regulating the activity of microtubule- associated proteins (MAPs) within the cytoskeletal network. The activation or deactivation of phosphorous groups controls the functioning of MAPs. Specifically, the phosphorylation of MAPs destabilizes microtubules by weakening the internal bonds that contribute to their structural stability [185]. The membrane potential of proliferative cells has a lower absolute value than that of quiescent neighbors [186]. Due to this, the malignant cells present low membrane potential [187]. Consequently, the VSPs influence the cytoskeleton in the permanently depolarized cancerous cells. The low level of cytoskeletal polymerization supports the proliferation and mobility of malignant cells.

The phosphorylation of MAPs not only affects microtubule stability but also plays a crucial role in the proper functioning of various ion channels, transporters, and vesicle movement within the cell. This mechanism enables the active modulation of intercellular electrolyte levels and protein connections in response to external electric fields. The dynamic stability of the system is governed by the Le Chatelier principle: a sudden change in membrane potential triggers phosphorylation, leading to an increase in potassium transport and the simultaneous suppression of sodium transport. This intricate process aims to restore the original membrane potential and effectively interacts with the cell proliferation process. The phosphorylation of VSP is energized by ATP. This energy consumption decreases ATP concentration, which

increases the depolarization of the cell membrane by suppressing the other, ATP-dependent active membrane transport of ions. The VSP has a role in anaerobic glycolysis and cancerous transformation when permanent stress conditions massively demand ATP. These conditions may activate the oncogenes, inhibiting apoptosis and producing high concentrations of stress proteins. This situation combats normal homeostatic regulation, so it is ideal for developing cancer.

The electric field polarizes the cytoskeleton's fibers [188], so it reorganizes the cytoskeleton in static (direct current, DC-field) [189] and dynamic (alternating current, AC-field) conditions [188]. The AC has the greatest influence at around 1 Hz [190], while in amplitude modulation of high frequency, it is optimal around 16 Hz (Adey window) [191]. This phenomenon exhibits resonant effects that can be described by stochastic resonance [192]. It assumes a bistable two-position state of voltage-sensitive phosphatase (VSP), like voltage-gated ion channels [193]. In the presence of a DC electric field, the membrane polarizes in opposite directions at different sides of the cell relative to the field vector. One side becomes hyperpolarized while the opposite side becomes depolarized. Depolarization triggers phosphorous hydrolysis, initiating cytoskeletal formation on the hyperpolarized side. However, in the presence of an AC electric field, both sides exhibit stochastic resonance and become hyperpolarized, leading to cytoskeletal reorganization. In the belt region perpendicular to the DC field, where there is no resonance, phosphorylation proceeds normally, creating a general phosphorous gradient in this region. The reorganization of the cytoskeleton is driven by specific forces, resulting in a pattern perpendicular to the pattern induced by the DC field.

The AC electric field induces resonance-like reorganization of the cytoskeleton, with a distinct peak at a specific frequency dependent on thermal noise [193]. It has been rigorously demonstrated that amplitude-modulated carrier frequencies can generate stochastic resonance, leading to various biological effects [193]. This phenomenon selectively stimulates enzymatic reactions, activates and deactivates voltage-gated ion channels, and reorganizes cytoskeletal polymerization processes [43] [45]. Moreover, the amplitude-modulated carrier can modify complex processes [44]. The modulating signal alone may also produce resonances. However, the requested low frequency alone, due to the impedance barrier of the skin, does not penetrate deep enough into the body. The 0.1 - 15 MHz high frequency has enough penetration to the human body and, as a carrier, delivers the signal for stochastic resonances. The optimal carrier frequency, as used in mEHT, may select the cancer cells with β, δ frequency dispersions [119] [194] and cause definitive apoptotic cell destruction of malignant cells [23].

Another approach is when the cytoskeleton polarization is optimized without modulation in high frequency (~0.2 MHz). These tumor-treating fields (TTF) target the cytokinetic "neck" with nonthermal polarization effects [195]. The electric field generated by TTF (Time-varying Tumor Treating Fields) exerts an influence on the polarizable microtubules and actin fibers within the cell. It has the potential to reorient these structures and, importantly, can impede the polymerization process of the cytoskeleton and hinder the assembly of the mitotic spindle [105]. The process does not use considerable SAR as mEHT does to arrest the proliferation by targeting the cytokinetic neck. This difference affects the treatment protocol. The TTF must be applied 18 hours/day for months, while mEHT with thermal optimizing has only 10 - 12 treatments for 60 min each.

4. Immune Effects

Immunogenic cell death is one of the main advantages of mEHT [196], which produces damage-associated molecular pattern (DAMP) [197] by extrinsic excitation of apoptotic pathways through various channels, including the TRAIL-R2 (DR5) death receptors [198] with the complex interaction of FAD + FASS molecules [41] [199]. The concomitant immune-stimulative treatments with dendritic cells [33] [66] and another stimulator [200], with the applied bioelectromagnetic forces by modulated RF signal [19] [44] [45] [201], improve the immunogenic processes. The immunogenic effects represent the oncology trend, especially oncological hyperthermia oncologic hyperthermia [18].

Due to the missing apoptosis, intensive proliferation is the assertive behavior of cancer. The mEHT, with its bioelectromagnetic excitations, promotes a massive preference for apoptosis against proliferative survival [23] [38] [202] [203]. Multiple excitable transmembrane proteins exist in the malignant cells (Figure 7(a)). The excitation could be thermal or nonthermal, but dominantly the synergy of the two, when the thermal process ensures the optimal conditions for the nonthermal excitation. The apoptosis may go through various signal pathways (Figure 7(b)).

The apoptosis uses an extrinsic pathway through Caspase-8 (Cas-8), an intrinsic pathway (Cas-9) finally, with Cas-3, to the programmed cell death (Figure 8). The caspase-independent way through apoptosis-inducing factor (AIF) is also activated, making it possible to execute the apoptosis when the caspase paths are blocked.

When one pathway is blocked by the malignant evasion of apoptosis, the other pathways may completely substitute the missing line.

The elevated levels of BAX observed in the affected cells [205] [206] further support the apoptotic effect induced by the treatment. The increased presence of BAX suggests the activation of apoptotic pathways and reinforces the notion that the treatment is triggering programmed cell death in the affected cells. The selective energy absorption of mEHT produces heterogenic membrane temperature, intensively heating the transmembrane proteins, including the TRPV channels. The TRPV promoted Ca^{2+} influx massively overloads the intracellular conditions with Ca^{2+} concentration. The thermal and nonthermal synergy of mEHT ensures the requested apoptotic level of Ca^{2+} overload.

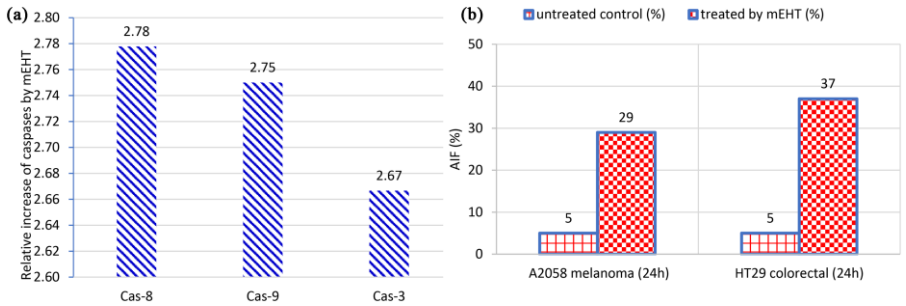


Figure 7. The critical protein involvements in apoptosis by mEHT. (a) The caspase involvements relative to the untreated samples (HepG2 cell-line in vitro [65]). (b) The AIF

percentages in the samples from in vivo xenograft experiments for A2058 melanoma [204] and HT29 colorectal carcinoma [205] tumors.

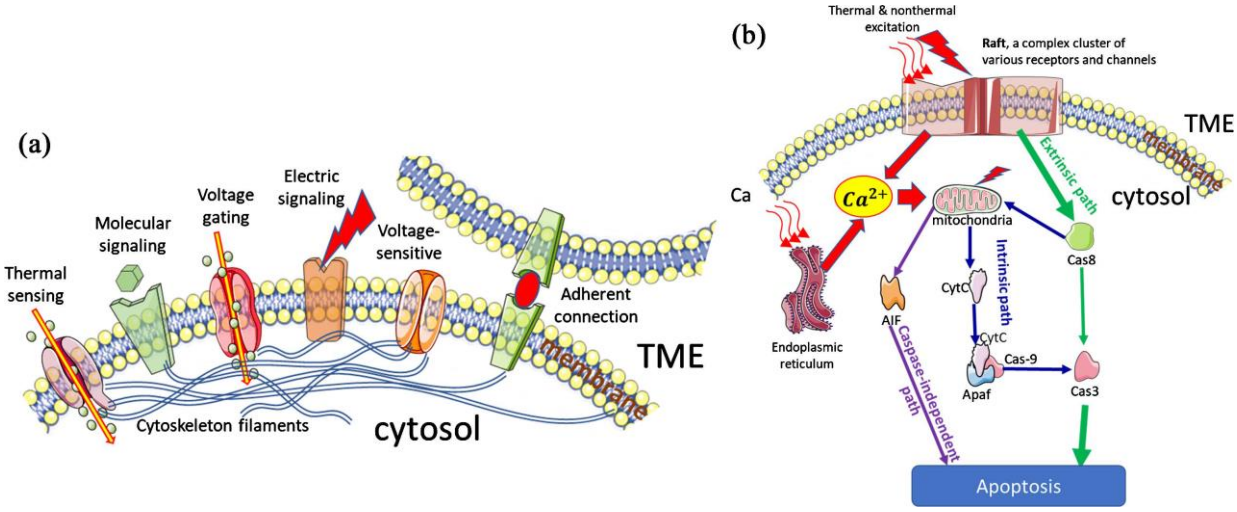


Figure 8. Forming apoptotic signals. (a) Multiple excitable transmembrane proteins accept the thermal and nonthermal effects from mEHT. (b) Multiple signal pathways are available, ensuring that a block in one can not terminate the apoptotic process.

The thermal and nonthermal stresses imposed on homeostatic control typically stimulate the production of chaperone stress proteins. The chaperoning HSPs are exhausted by mEHT and cannot meet the chaperone function to protect the cells against apoptosis [207]. A part of HSP70s is secreted on the membrane [119], and this localization [208] promotes apoptosis [209] and has a vital role in the membrane "fluid' to keep it functional [210].

While cancer has strong proliferation, it is weak in its loneliness. The cellular autonomy is a weak side, which offers a correction possibility. Immune surveillance is critical in attacking the weakness of malignancy and guaranteeing homeostatic balance. The counterbalance of the evasion of immune effects by malignant cells needs local and systemic activity, which rebuilds the standard healthy conditions. The autonomy of malignant cells shows the breaking of adherent and junctional connections with the neighboring cells, substantially modifying the homeostatic electrolyte composition and concentration in the TME [26], which offers an electric selection factor [211] [212]. The reorganization of the cytoskeleton by nonthermal electric polarization of mEHT promotes the form (β-catenin + E-cadherin) complexes [65] [213], giving a possibility to reestablish the lost adherent connections and fix the cancer cells in their position, block the dissemination.

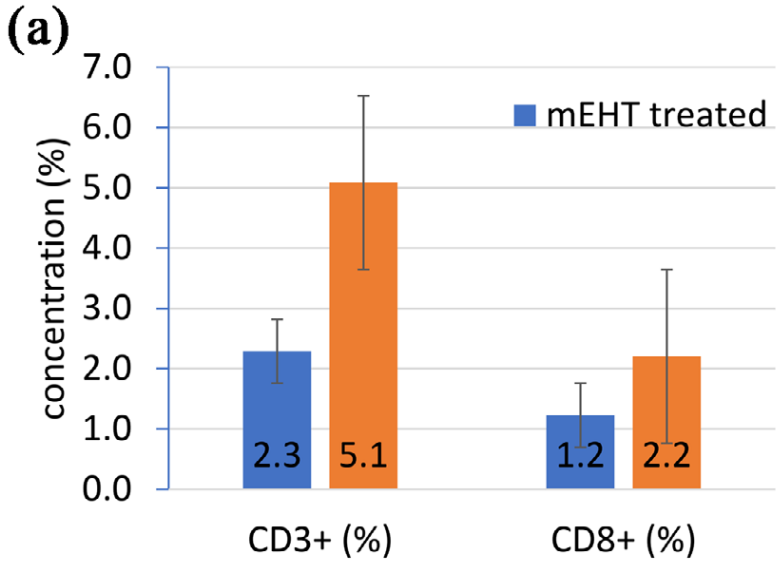
Immune control creates an additional possibility to block the cellular autonomy of malignant cells by destroying the freely moving cancer cells. The great and unique advantage of tumor-specific immune activity is its ability to find and eliminate distant micro and macro metastases locally and systemically. The frequently applied CAR-T method has the same purpose [214], preparing tumor- specific T-cells from the patient's blood sample. Immune effects integrate the TRP channels for multiple purposes [215]. All immune cells, including NK cells [216], T-cells [217], and dendritic cells [218]), have TRPV1 channels with significant functions. The CD34+ hematopoietic stem cells express TRPV2, which is also expressed in

granulocytes, monocytes, and CD56+ natural killer cells and orchestrates the Ca^{2+} signals in CD4+ and CD8+ T-cells and CD19+ B lymphocytes [219]. Moreover, TRPV1 modulates macrophage-mediated responses [220].

The adaptive immune reaction was measured, detecting significant development of DC cells (S100) for maturation (antigen-presenting) CD3+ CD4+, CD8+ T-cells and suppressing Treg cell-population (Foxp3) (Figure 9) [33].

The TRPV1 and TRPV4 channels have been implicated in T-cell activation and the production of effector cytokines. These channels play a role in suppressing the release of tumor necrosis factor (TNF) and interleukin-2 (IL-2), which are important immune signaling molecules involved in inflammation and immune responses [217]. Furthermore, the TRP channels have a critical role in controlling phagocytosis, the production of chemokines and cytokines, and cell survival [221].

The HSPs are not less important in cell fate and immune activity than TRPs. The thermal and nonthermal stress combination overloads the malignant cells with chaperoning HSPs, which have much more function than only chaperoning, depending on their position in the cell [210].



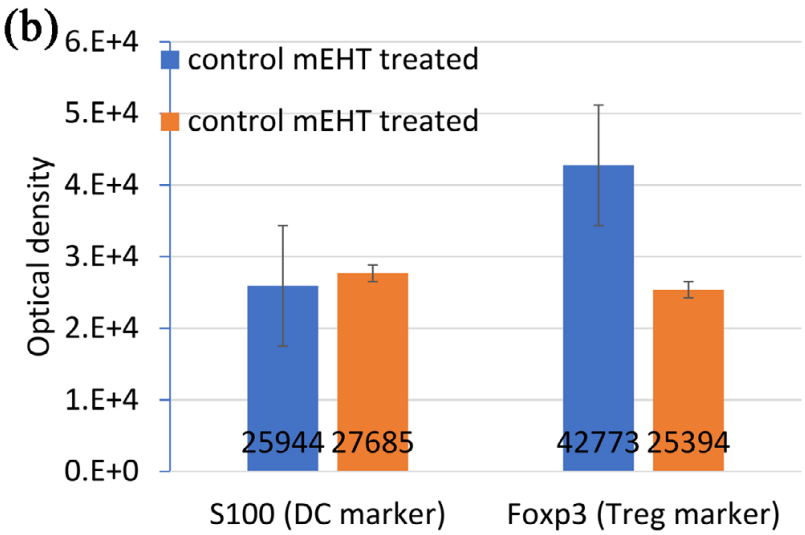


Figure 9. The relative development of the critical immune-surveillance cells in SCCVII allograft after mEHT [33]. Measured with (a) flow cytometry and (b) optical density.

To initiate the apoptotic process, the mitochondrial heat shock proteins (mHSPs) must first bind to a complex formed by tumor peptides [222]. The membrane-localized HSP70s [223] are mainly localized in the rafts [224] and activate the NK cells in the immune response [204] [225].

A part of the HSPs may leave the cells and become extracellular [226]. Their importance in building up an appropriate immune response is crucial. These released molecules deliver tumor-specific information for antigen presentation to develop antitumoral immune reaction [115], which process attracts much attention in studies of the overall immune reactions of the bio-systems [227] [228]. The antigen-presenting cells (APCs) develop the tumor-specific CD8+ killer and CD4+ helper T-cells, which are delivered by the bloodstream and combating with the cancer cells in the entire body (abscopal effect) [229]. The local treatment developed a whole-body effect by mEHT [230] (Figure 10). The in-situ and real-time production of tumor-specific immune activity is the advantage of mEHT [231].

Significant development of the DAMP molecules characterizes the results of mEHT treatment. The abscopal effect of distant, untreated tumors was observed when immune stimulation was added to the protocol (Figure 11) [200].

The thermal and nonthermal effects work in synergy to produce the in-situ immune effects with mEHT. In the thermal aspect, the relative mild tissue temperature is crucial for immune development. The enhanced temperature eases the enzymatic processes and increases the molecular reaction rate, but a higher temperature than 40°C blocks the activity of the immune cells [232] and so does not allow the real-time processes for APC prepared by immunogenic cell death [231]. Furthermore, the > 40°C temperature paralyzes the NK cells to attack the cells with the marks of the HSP70s on their membrane [233]. The immune cells may restore their activity with elapsing time [234] or by bloodstream replace them from the non-treated parts of the body, but the real-time processes with the simultaneous reactions vanish. The heterogenic heating of mEHT with the high temperature of rafts and simultaneously mild of

the tumor microenvironment (TME) solves the contradictory demands of the hyperthermic temperature range and the immune requirements.

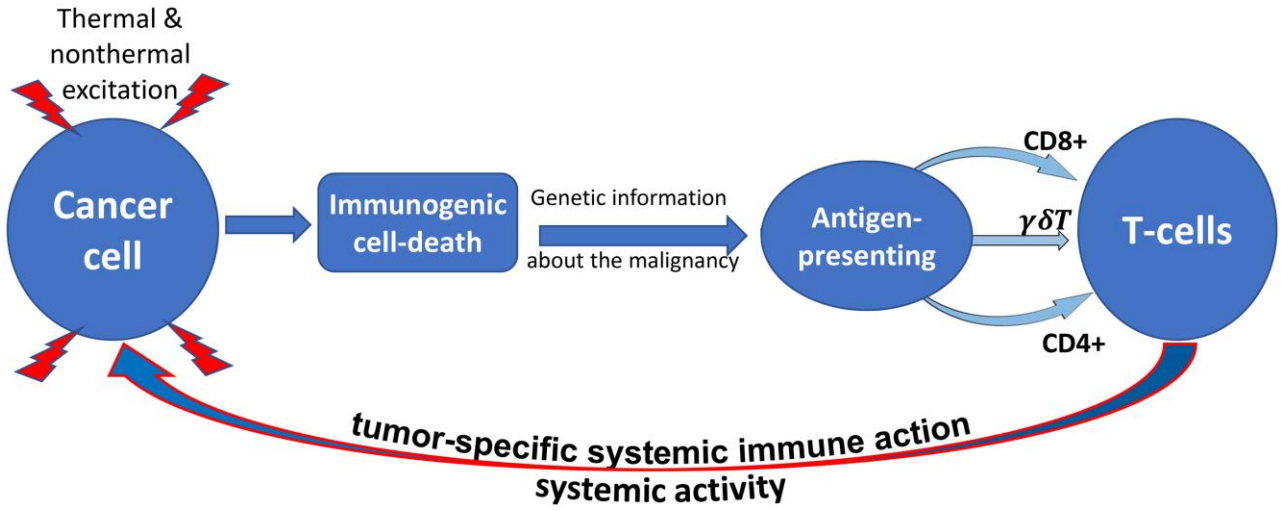
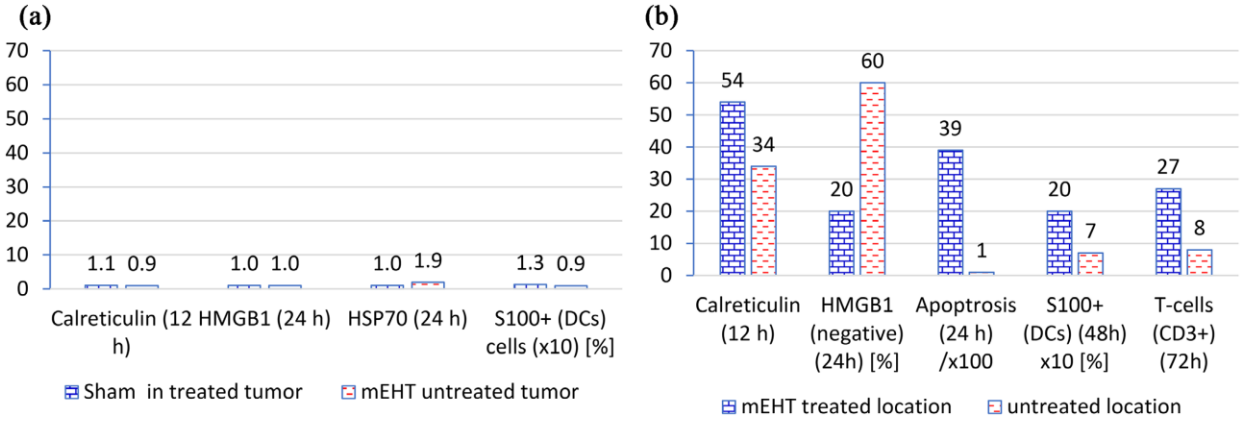


Figure 10. The thermal and nonthermal processes develop immunogenic cell death. The genetic information used by antigen-presenting produces tumor-specific T-cells. In this way, the adaptive immune machinery starts a systemic attack of malignant cells all over the system.



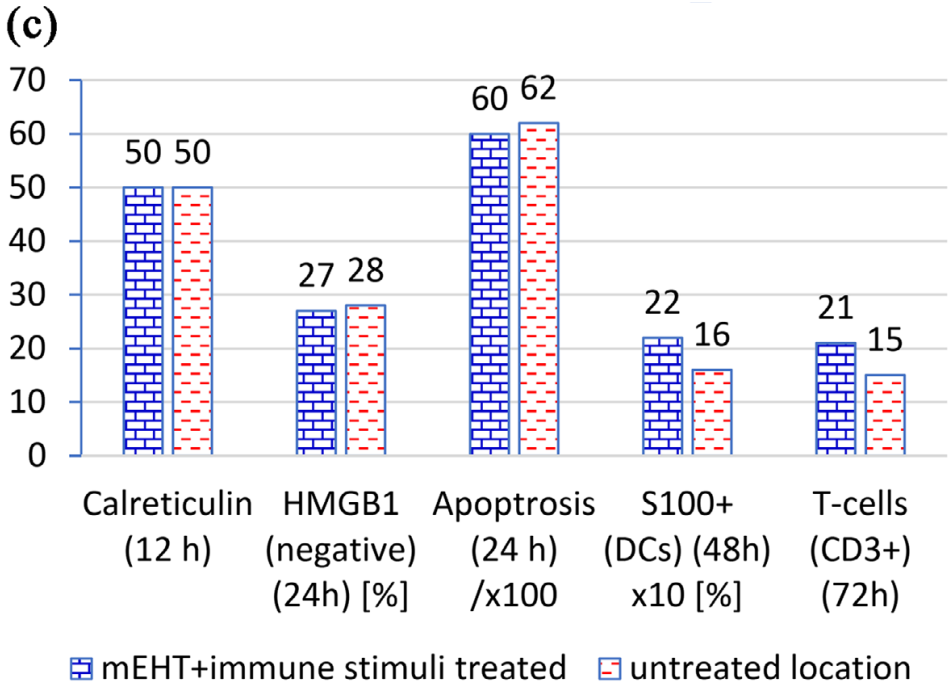


Figure 11. Development of DAMP molecules in colorectal allograft preclinical experiment of C26 tumor [22, 200]. (a) The sham treatment and untreated tumor on the same animal had treatment at a distant location. (b) Development of the DAMPs in the treated and untreated tumors on mice. (c) Development of DAMPs in treated and untreated locations when immune stimulants were added to the protocol.

The nonthermal processes in mEHT are also essential for the abscopal effect. The excitation of the TRP channels sensitizes the immune lymphocytes [215] and promotes apoptosis [101], helping with the ICD processes. The substantial nonthermal stress exhausts the evasion of apoptosis in cancer cells and produces transmembrane HSP70, which also supports apoptosis [209]. It activates ligands for NK cells [208], forming an innate immune response [235], especially in tumor cells [223]. The well-forming temporal order by mEHT of membrane secretion calreticulin, the extracellular release of HMGB1 and HSP70 developing the APCs for an active adaptive immune response [231].

Numerous preclinical studies prove the specialties of mEHT (Figure 12). A recent review summarizes the results [236].

Based on the regulatory conditions the mEHT method received the necessary certifications and numerous clinical trials were performed in different hospitals in various countries (Figure 13). Some protocols allow geriatric and pediatric considerations, too. A recent review summarizes the results up to 2019 [242].

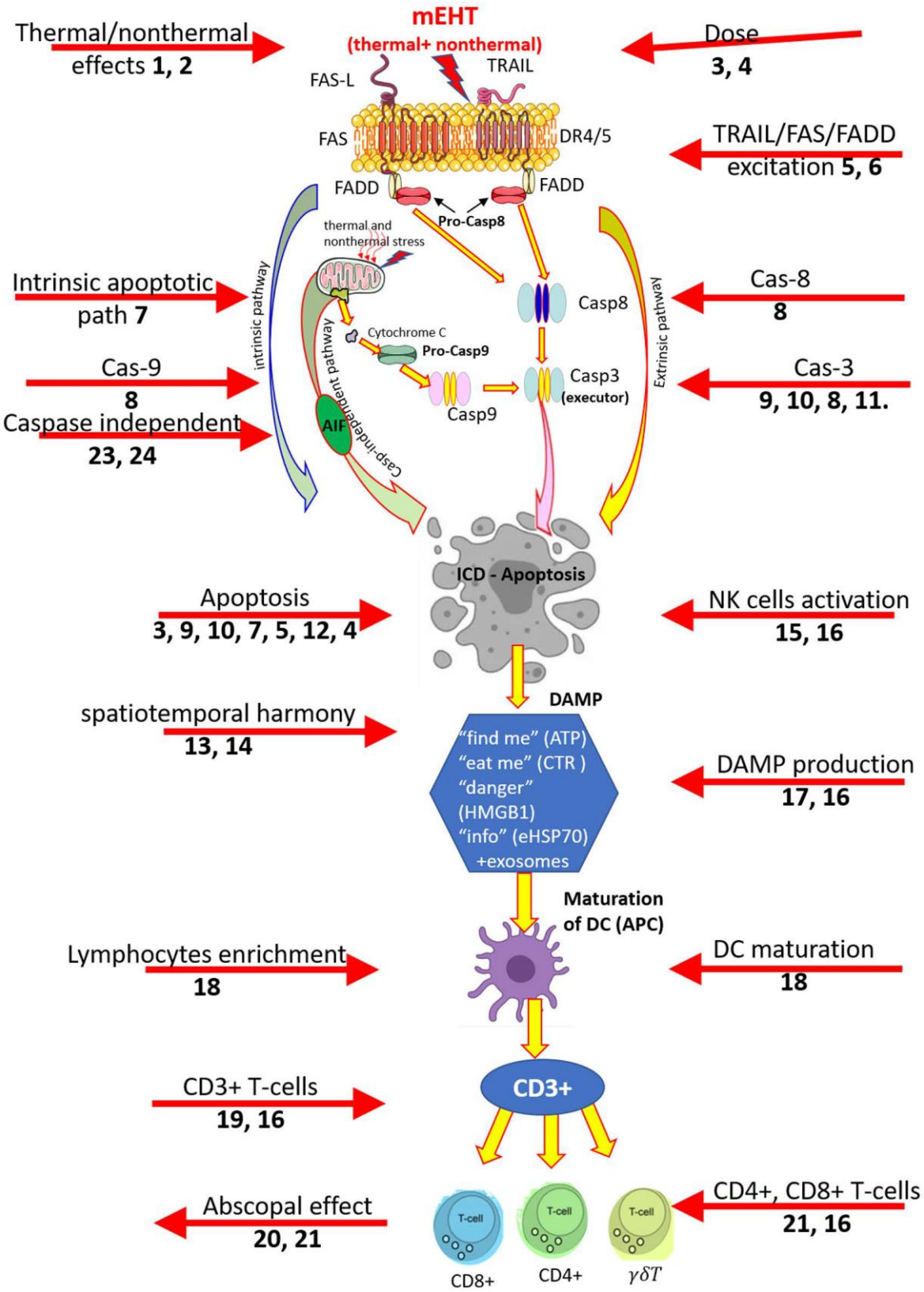


Figure 12. Some preclinical experiments with mEHT method. The numbers refer on the references: 1. = [42], 2. = [237], 3. = [238], 4. = [94], 5. = [202], 6. = [198], 7. = [239], 8. = [65], 9. = [207], 10. = [203], 11. = [240], 12. = [64], 13. = [207], 14. = [206], 15. = [204], 16. = [32], 17. = [205], 18. = [66], 19. = [241], 20. = [200], 21. = [33], 23. = [205].

5. Conclusion

The modulated electro-hyperthermia focuses on the nonthermal and thermal effects synergy when the thermal component provides optimal conditions for the nonthermal electric molecular excitation. In this review, we concentrate on the role of the ionic channels as TRPs, VSPs, and voltage-gated channels in the selective antitumoral processes. These transmembrane compartments primarily promote the Ca^{2+} and the H + influxes, interact with the cytoskeleton and are involved in the apoptotic signal pathways. The DAMP forming TRAIL-FAS-FADD excited extrinsic apoptotic signal combined with the Ca^{2+} induced apoptosis ensures a "gentle" distortion of the malignant cells, which, together with the fully functioning other DAMP molecules, uses the membrane secreted and extracellularly released HSPs with the exhaustion of their intracellular chaperoning to form APCs. The innate and adaptive tumor-specific immune activity appears by the membrane HSP70 and the APC-produced killer and helper T-cells. The
bloodstream-delivered T-cells attack the cancer cells all over the body (abscopal effect), so the immunogen processes transform the local mEHT to systemic.

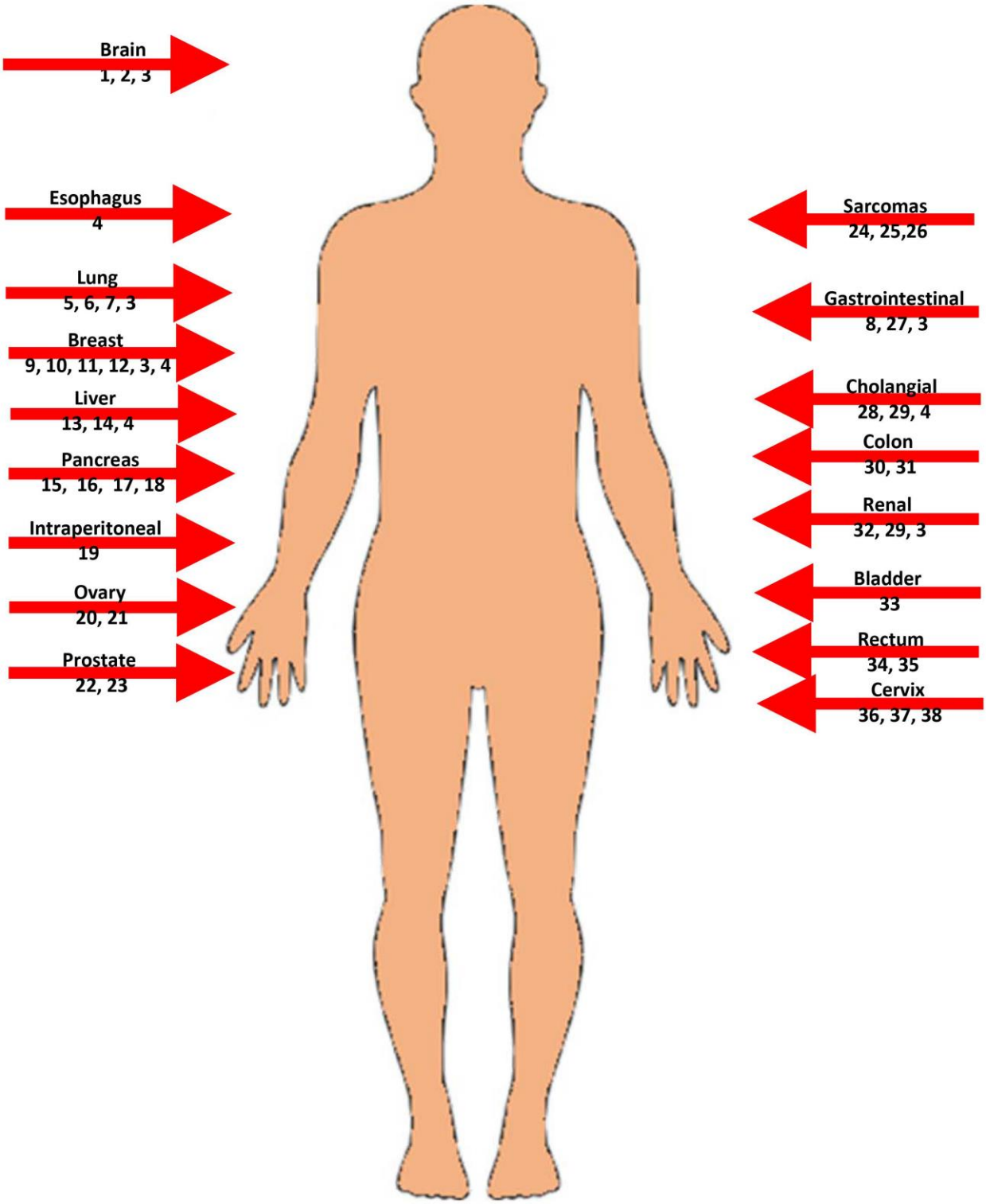


Figure 13. The clinical studies with mEHT. It contains various levels of evidence including case reports and phase II/III trials. The numbers refer to the references: 1. = [243], 2. = [244], 3. = [245], 4. = [246], 5. = [247], 6. = [248], 7. = [249], 8. = [250], 9. = [251], 10. = [252], 11. = [253], 12. = [254], 13. = [255], 14. = [256], 15. = [257], 16. = [258], 17. = [259], 18. = [260], 19. = [261], 20. = [262], 21. = [263], 22. = [264], 23. = [265], 24. = [266], 25. = [267], 26. = [268], 27. = [269], 28. = [270], 29. = [254], 30. = [271], 31. = [272], 32. = [273], 33. = [274], 34. = [275], 35. = [276], 36. = [277], 37. = [278], 38. = [20].

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Conflicts of Interest

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

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