
PRECLINICAL VERIFICATION OF MODULATED ELECTRO-HYPERTHERMIA

PART I. – IN VITRO RESEARCH

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ABSTRACT

Modulated electro-hyperthermia (mEHT) targets tissue's natural electric and thermal heterogeneities to heat the cancer cells selectively. The applied 13.56 MHz radiofrequency (RF) is a carrier of the low-frequency modulation. The high-frequency part was chosen to select the malignant lesion using the specialties of the tumor: the higher conductivity and dielectric constant of the tumor than its host. The electric field selects the tumor, and the low-frequency amplitude modulation polarizes and excites the transmembrane proteins of the malignant cells. The dominant absorption of the energy by the microscopic clusters of the membrane rafts acts like nanoparticle heating. Exciting the membrane produces various apoptotic signals. The processes were modeled using silico and phantom experiments, which proved the concept. The preclinical verification was made in vitro and in vivo, and in the end, clinical proofs validated the method. Our objective is to follow all the development steps from the laboratory to the clinics in a trilogy of articles. This present is the first part, which deals with in silico, phantom, and in vitro research.

KEYWORDS

Modulated Electro-Hyperthermia, mEHT, In Silico Calculations, Phantom Measurement, In Vitro Experiments, Thermal Effects, Nonthermal Processes, Apoptosis

1. INTRODUCTION

Cancer therapies have rapidly developed in the last decades. All classical treatments like chemotherapy (ChT), radiotherapy (RT), and surgery (Op) went through intense variation. The new, highly effective drugs and the personalized medication renewed the ChT. The emerging immuno-oncology (IO) introduced a new concept of cancer elimination with the help of the body's regulative capabilities instead of cytotoxic drugs. The tomotherapy and the ion-beam therapies made a breakthrough in RT, while in surgery, the high-precision, remotely operated surgery devices and new laparoscopic solutions represent vital developments. Hyperthermia also had numerous new developments and improved technical realizations, but its medical recognition remained behind the conventional treatments. Conventional oncological hyperthermia intends to heat the tumor as much as possible. However, bioelectromagnetics and immunology are game changers in oncological hyperthermia, far beyond the task of simple heating. The hyperthermia process had changed. The method thermally induces various processes governed by bioelectrodynamics. The thermal effect is a part of the energy that heats and appears in the temperature increase. Another energy part excites electric and chemical processes that are well-organized in space and time, choosing the optimal cell death to eliminate the malignancy. Cell destruction does not depend only on temperature, it could be guided by nonthermal processes, knowing that the living structures are more chemical than thermal "machines." Oncological hyperthermia is a method to kill malignant cells by absorbing energy characterized by a specific absorption rate (SAR). The SAR acts synergistically with thermal and nonthermal effects. The critical part of energy absorption is how the thermal effects ensure optimal conditions for the nonthermal molecular processes. Optimal oncologic hyperthermia sensitizes certain complementary methods like radio (RT) and chemotherapies (ChT). In hyperthermic effects, in

addition to the thermal impact, we consider the nonthermal processes, which cannot be produced thermally alone but use thermal conditions to optimize the triggered reactions.

Thermal homeostasis, a fascinating process that regulates the body's temperature compared to a set point in the hypothalamus, is at the heart of our research. It seeks to restore the baseline condition with a nonlinear regulation, including improving electrolyte transport in the heated area and intensifying various physiological mechanisms supporting its forceful control. Living organisms are not thermal 'machinery' but rather chemical entities. The thermal effects are conditional, and the ignited reactions are chemical, with their reaction rate being sharply temperature-dependent. Interestingly, nonthermal changes can be electromagnetically promoted, adding another layer of complexity to our study.

The increasing target's temperature promotes the nonthermal "chemical machinery" [1] [2]. These effects must be used in synergy, which is realized by the well-chosen modulated radiofrequency (RF) electromagnetic effect in the mEHT technique [3]. The thermal and nonthermal effects form a synergy (Figure 1) and determine the actual processes together. The thermal component provides the appropriate temperature of the tumor microenvironment (TME) by heating the membrane rafts [4]. Another general thermal action affects the extracellular matrix (ECM) and a part of the TME. This acts mechanically and molecularly [5], accompanying the thermal absorption of transmembrane protein clusters.

The incorporation of energy happens at clusters of transmembrane proteins [6], [7] [8]. The nonthermal component excites the membrane receptors of the cells. The well-chosen radiofrequency electric current can deliver energy for molecular excitations involving various ionic and molecular interactions [9]. The process only has a moderate thermal effect and excites the molecules or structures that fit the applied resonant conditions.

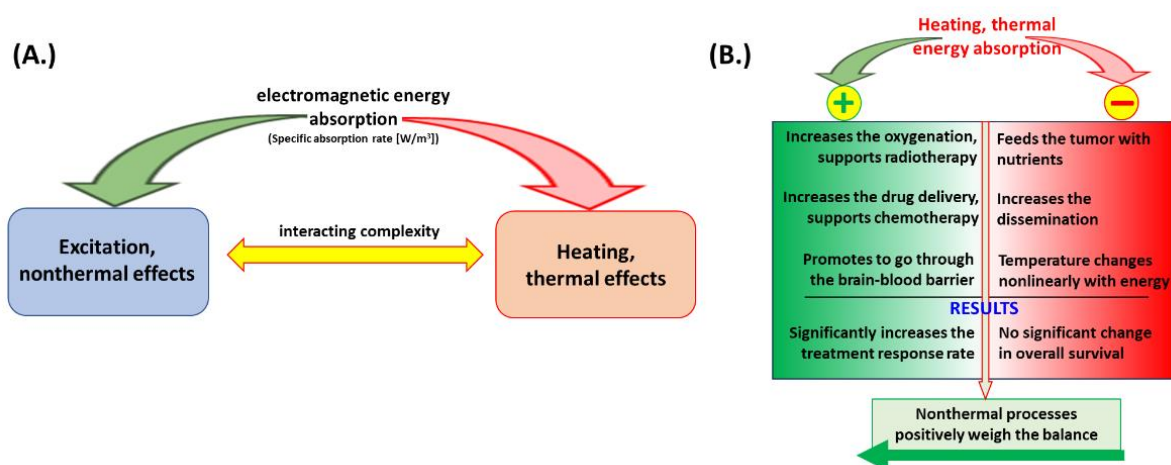


Figure 1. The synergy of the thermal and nonthermal effects. (A.) The division of the electrodynamic energy to thermal and nonthermal components and its complex cooperation. (B.) The thermal energy absorption balances between the pro and antitumoral processes. The nonthermal component tilts the balance towards antitumoral effects.

Researchers in conventional oncological hyperthermia usually share the opinion of the editorial comment of the European Journal of Cancer in 2001: the biological effects are impressive, but physically, the heat delivery is problematic. The famous formulation blames physics: "The biology

is with us, the physics are against us" [10]. Later, it was slightly modified, blaming the physiology against the well-defined hyperthermic goal, the high temperature [11]. Recently, the opinion of blaming was questioned [12]. This paper aims to present the preclinical data of a novel hyperthermia method called modulated electro-hyperthermia (mEHT), which directly presents the biophysical and physiological support in the hyperthermic elimination of malignancy. This article presents the preclinical data of a novel hyperthermic method, modulated electro-hyperthermia (mEHT), which directly demonstrates the hyperthermic destructiveness of malignant tumors with intensive support for the biophysical and physiological processes.

2. METHOD

The modulated electro-hyperthermia (mEHT) electric field. It is a kind of hyperthermia that targets tissue's natural electric and thermal heterogeneities and, with these particular properties, identifies and destroys cancer cells with definite carrier frequency and its optimally chosen modulation [2] [3]. The applied 13.56 MHz radiofrequency (RF) is a high-frequency carrier of low-frequency modulation [1]. The high-frequency part was chosen to select the malignant lesion using the specialties of the tumor: the higher conductivity and dielectric constant of the tumorous cancer than its host. The frequencies are chosen for the optimal use of the frequency dependence of natural heterogeneities [3] [4]. The electric field selects the tumor, and the low-frequency amplitude modulation polarizes and excites the transmembrane proteins of the malignant cells. The primary guidance of mEHT is biophysical using updated bioelectromagnetic considerations. The intensive proliferation of tumors is accompanied by a high metabolic rate, which produces extensive ionic density and higher electrolyte content in the tumor microenvironment (TME). Consequently, the tumor has high electric conductivity, which could guide the applied radiofrequency current to flow through it. Furthermore, the current may differentiate the autonomic malignant cells from the networked healthy counterparts in the heterogenic tumor mass. These selection facilities allow a heterogeneous, targeted effect instead of the intention of the overall isothermal heating of the tumor mass [13]. The essential step is the energy absorption of the transmembrane proteins in cancer cells and triggering intracellular signals driving immunogenic cell death, a special kind of apoptosis [14]. The carrier frequency modulation supports choosing the appropriate intracellular signal pathways [15] [16]. Time-fractal modulation endorses the selection of malignant cells [6], heating them locally to the hyperthermia temperature to induce cellular changes in the targeted cells by thermal and nonthermal mechanisms [1]; Figure 2 The thermal component of the absorption heats the selected membrane rafts, which is the source of the temperature of the tumor, as is standard in heterogenic seeds or nanoparticle heating processes. In contrast, the nonthermal processes excite signal pathways for programmed cell death [17]. The principle of the nonionizing radiation used by mEHT is very similar to the principle of ionizing radiation (radiotherapy, RT). The RT isodose actively concentrates on DNA damage using homogeneous radiation to produce heterogeneous (nanorange) effects to induce apoptosis. The mEHT is also a radiation isodose but nonionizing in the RF range and focuses in nano range on transmembrane proteins exciting apoptotic signals.

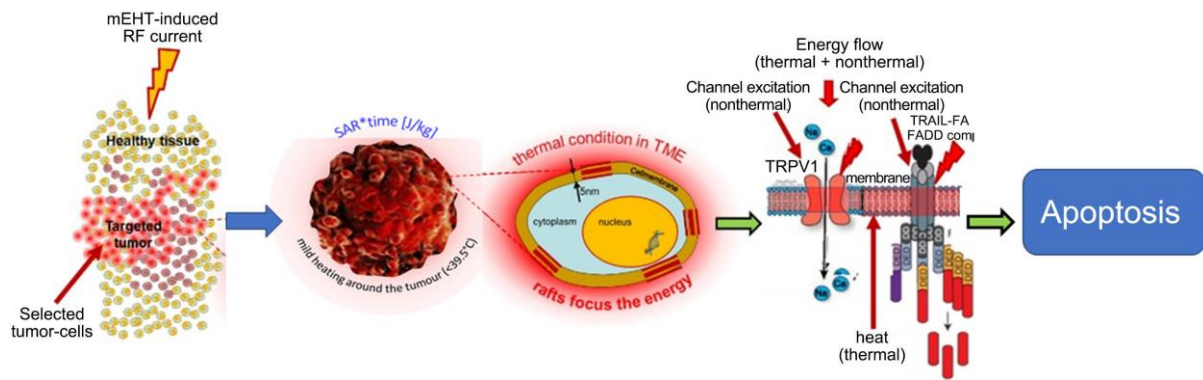


Figure 2. The transmembrane proteins of malignant cells absorb the energy in thermal and nonthermal forms. The nonthermal effect gives the primary drive for the apoptotic signal pathway (see below in the results).

The technical realization of the mEHT uses numerous biophysical concerns [13] [18] [19]:

- 1) The radiofrequency (RF) carrier frequency is 13.56 MHz. It is a freely applicable ISM band [20], so it does not need shielding.
- 2) The energy is capacitively coupled but avoids isothermal heating; it does not use the plane-wave method. The capacitively coupled electric field has precise impedance matching, mimicking the contact electrode conditions [21]. The precise impedance matching produces negligible reflected power (~1 W) and behaves like galvanic contact with the skin.
- 3) The method automatically selects the cancer cells, considering the well-measurable biophysical differences between the malignant and healthy tissues [22].
- 4) The maximum adequate output power of mEHT is limited to 250 W, less than for isothermal heating, but high enough to select and excite the membrane rafts of the malignant cells [1], and sensitize them to the radio [23] [24] and chemotherapies [25] [26] in clinical applications.
- 5) The modulation spectrum is a low-frequency time-fractal [27], described by fractal physiology [28] [29], which agrees with the homeostatic molecular temporal balance [15]. mEHT extensively uses the modulation technique to identify fractal structures in space and time (dynamics) [15]. It helps the temporal arrangement of the homeostatic regulation on the molecular level.
- 6) The membrane rectifies (demodulates) the signal [30] [31], which acts in the low-frequency range.
- 7) The correct impedance matching provides an appropriate electric field that ensures the current density (j).
- 8) The modulated j -current density actively produces both the thermal and nonthermal effects.

- 9) The patient is interactively connected to the electric circuit, like a discrete element of an RF circuit. This solution allows the patient to be controlled in real-time because the treated tumor is actively sensed and targeted as part of the tuned electric circuit [32].
- 10) The excited transmembrane ion channels function like RF rectifiers and low-pass filters [33].

Preclinical research is a long, multistep investigation process. It starts with the concept, proven in silico research and verified by phantom measurements. Phantoms lack more complexity than living organisms. This model verifies only the thermal effects. The research continues with in vitro experiments, which miss the physiological regulation processes we could model in the next step in vivo. All models give new knowledge, which appears as feedback for the previous research steps, which continues until the data show enough accuracy for the proof of the concept, the advantages and disadvantages, and safety to start the clinical phase Figure 3.

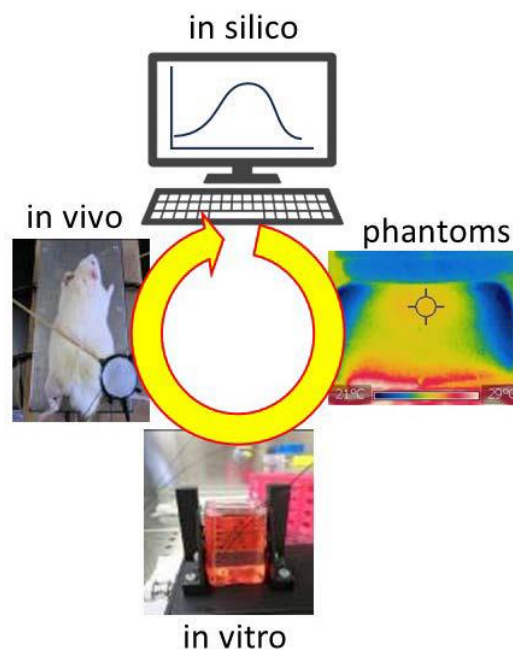


Figure 3. The preclinical methods have intensive feedback from in silico to in vivo through phantoms and in vitro experiments until all information is harmonized.

The in silico calculations are primarily based on bioelectromagnetic and thermal modeling of the living tissues, applying the commercial, highly specialized software (Computer Simulation Technology (CST), Darmstadt, Germany), and the various solutions of the Pennes bioheat equation [34]. The first period with growing temperature was calculated, which is too early to consider the convection term in the equation, considering that the physiological reactions have a considerable time lag. However, in some cases, the convection term was considered using the linear approach.

In further research, when long-time heating is considered, the new approach [35] could be considered. The phantom measurements used tissue-equivalent materials or meat tissues, mimicking the actual thermal situation in living objects. The phantom models describe the thermal effects, but due to the essence of the phantom construction, those cannot follow nonthermal effects.

The in vitro and in vivo research are more complex. These methods have a lot in common, but the physiological regulation in vivo shows the temporal development of the synergy of thermal and nonthermal effects during energy absorption. The feedback mechanisms differentiate the temporal progress in the two kinds of experiments. The thermal processes have a complex nonlinear interaction with the homeostatic regulation, which tries to keep thermal homeostasis. The hypothalamus receives the thermal signal (primarily from TRP receptors) and acts to reduce the temperature. The principal effects are the blood flow with vasodilation and the sweating trying to cool with the evaporating process. The thermal regulation balances the incoming energy, which nonlinearly fluctuates in equilibrium Figure 4. incoming energy [36]. The fluctuation has various physiological components, including the opposition sensory mechanisms [37] and the various relaxation times of the different processes. The primary relaxation times could be measured with NMR [38], and the complex processes can be calculated using multiple physiological measurements [39] [40].

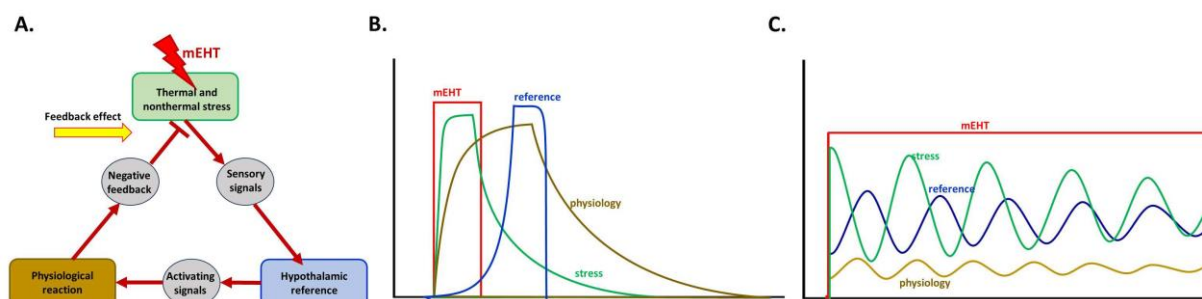


Figure 4. Effect of timing in homeostatic control. (A.) The feedback mechanism of regulation. (B.) The developments of the various signals. (C.) The signal modification during the mEHT process.

The in vitro cellular mechanisms have a short chemical reaction (≈ 1 s) timescale while its molecular developments take a long time (> 1 h). This feedback synergizes with in vivo development, and the observed signal is $1/f$ noise [41] [42]. The in vivo experiments are fundamentally different from the human clinical observations, but the allometry could help to emphasize the similarities [43]–[45].

The limitation of all such in silico calculations is the extensive complexity of living organisms. The homeostatic control includes multiple negative feedback regulations, which can be followed only in a simplified mathematical approach.

3. IN SILICO CONSIDERATIONS

The most important in silico publications and their results are shown in Table 1. The apoptotic signal by the mEHT excited membrane receptors and the apoptosis by the single or double-strand breaking of DNA by ionizing radiation are similar in their molecular selection principle. Nevertheless, despite conceptional similarities, the two methods have an essential difference: the nonionizing electromagnetic processes have massive thermal effects as the basis of hyperthermia; on the contrary, the thermal component is minimal in ionizing radiation, and it is primarily an unwanted side effect of radiotherapy. The final goal is shared: destroy the tumor cells with well-chosen molecular effects.

Table 1. In silico considerations in preclinical research.

Idea, considerations	Method	Findings	Conclusion	Ref.
The temperature development is stochastic.	Modified Pennes equation.	Temperature develops by Weibull distribution.	Dynamic, nonlinear development of temperature.	[46]
The correct dosing of hyperthermia needs chemical considerations.	Chemical transitions with multiple Arrhenius fit.	The correct dose needs transition-state theory (Eyring).	The presently applied CEM43Tx dose has to be modified.	[47]
Electromagnetic resonances in oncotherapies.	Self-organizing considerations of homeostasis.	Stochastic resonance describes the enzymatic and collective processes well.	The optimal synergy of thermal and nonthermal components needs modulation.	[9]
The membrane rafts robustly absorb energy.	Electromagnetic description of the rafts and their environment.	The membrane rafts have a highly tremendous specific absorption rate.	Membrane rafts are selectively heated, and their temperature is exceptionally high.	[4]
The energy absorption of the extracellular matrix makes special conditions.	Thermodynamical considerations with Onsager's theorem.	The temperature gradient is enormously high on membranes, increasing the intracellular pressure and diffusion conditions.	The tissues have nonuniform temperature development, and the heterogeneity determines the hyperthermia effects.	[5]
Study the dosing and depth effect.	Impedance matching coupling.	Impedance matching extends the penetration depth.	The coupling technique of the electromagnetic field is an essential factor in the treatment's success.	[48]
Critical analysis of the available literature on nonthermal effects.	Biophysical and electrophysical model for nonthermal membrane effects.	Transmembrane ion channels rectify and low pass filtering. Resonances cause membrane depolarization.	Exists nonthermal antiproliferative effects of mEHT. It may improve future treatments in oncology.	[33]
Clarifies how the amplitude modulation interacts with thermal effects.	Bioheat transfer equation for tumor model.	Low-frequency amplitude modulation increases extracellular absorption.	The mEHT energy absorption has only subtle thermal and mostly nonthermal effects.	[49]
How the modulation affects the glycolysis.	Pennes equation with stochastic solution.	The modulated electric field affects the chemical reaction rate similarly to the temperature.	The modulation of the electric field is directly connected to thermal effects.	[50]
Time fractal modulation as a factor of the self-organized homeostatic synchrony.	Fluctuations and stochastic resonance in biosystems.	1/f noise production.	Environmental noises synchronize the stochastic processes, but the system has noiseless internal signal communications.	[15]
How does the time-fractal amplitude modulation destroy the cancer cells?	Thermal and nonthermal synergy with homeostatic feedback.	The time-fractal amplitude modulation could destroy the cells out of the healthy cellular network.	The modulation of the RF-carrier frequency, with the homeostatic 1/f signal, selectively targets the malignant cells.	[15]
Clarify the role of the heat shock proteins.	Study the conditions of HSP development.	The HSP's Intra or extracellular (or membrane) location determines their actions.	The immunogenic actions depend on the HSP info delivery in the extracellular matrix.	[51]

Study of immunogenic effects of mEHT.	Immunogenic cell death and damage-associated molecular pattern.	The mEHT may induce immunogenic effects by innate and adaptive immune processes.	Tumor-specific immune reaction products abscopal effect.	[14]
Bioelectromagnetism of angiogenesis.	The electric field induces polarization and current.	The mesenchymal ↔ epithelial transition depends on the electric field.	The appropriate electric field may influence the mesenchymal ↔ epithelial transition.	[52]
Angiogenesis has allometry.	Allometric scaling on a self-organized basis.	Allometric relation exists between tumor mass and cell death.	The inflammatory feedback could explain some unsuccessful tumor treatments.	[45]
Comparison of capacitively coupled methods.	Comparison of plane wave and impedance matching capacitive coupling.	The impedance matching has better performance than the plane wave coupling.	Self-selection is a good approach that could have immunogenic effects.	[21]
Model simulation of applicators for preclinical experiments of mEHT.	Temperature and SAR calculation for small electrodes (Ø10mm) developed for in-vivo murine models.	The SAR is strongly inhomogeneous. The different tumor growth stages have different temperature and SAR profiles.	The SAR is extreme in heterogenic places. The model verifies the nonthermal effects.	[53]

The nonthermal effect in mEHT involves “when, under the influence of a field, the system changes its properties in a way that cannot be achieved by heating” [54], like feeding cannot be replaced with the same heating energy. The nonthermal component excites the membrane receptors of the cells [2]. The thermal and nonthermal (electric) effects have similar Arrhenius-like exponential reaction rates [50]. The well-chosen electric current can deliver energy for molecular excitations involving various ionic and molecular interactions [46]. The process has a conditional thermal effect and excites the molecules or structures that fit the applied resonant conditions [9]. Two essential effects are considered for selection: thermal absorption and nonthermal excitation. The thermal component provides the appropriate temperature of the TME by heating the membrane rafts [4] Figure 5. which are nano-range clusters, widely vary by size 10 – 100 nm [55]; 25 – 700 nm [56]; 100 – 200 nm [57]. The rafts trigger intracellular signal pathways and determine cellular processes [58]. The temperature of these nano-units increases by the square of their radius [59]. The thermal process of mEHT heats the TME and the rafts, which serve for thermal and nonthermal processes. The RF current flows through the tumor and heats the extracellular electrolytes. Due to the high ionic concentration, the TME absorbs more energy than the ECM in more distant regions. The energy analysis of the heating differences explains how this effect contributes to cell-killing mechanisms [5].

Another general thermal action affects the extracellular matrix (ECM) and a part of the TME. This acts mechanically and molecularly [5], accompanying the thermal absorption of transmembrane protein clusters. The point selection shows of ~1 MW/kg absorbing possibility in 100 µm sphere [12]

[49]. The rafts absorb an average. $\sim 350 \frac{\text{kW}}{\text{kg}}$ [5], which fits the interval of nanoscopic heating 100 – 500 kW/kg [60]. The selected rafts’ temperature is over the tissue’s thermal averaging. The ECM also absorbs energy from RF current. The TME is especially heated due to the locally high ionic concentration and dielectric permittivity. This thermal process induces a temperature gradient

between the TME and the cytosol through the membrane. The energy analysis reveals how the gradient contributes to cell-killing mechanisms [5].



Figure 5. The particular nano effects in the cell membrane calculated by Computer Simulation Technology (CST) [4]. The point at touching two cells has concentrated energy absorption

$(\sim 2 \cdot 10^8 \frac{W}{m^3})$, while the cluster of transmembrane proteins (membrane rafts) absorbs more than double $(\sim 5 \cdot 10^8 \frac{W}{m^3})$ power, which promotes apoptosis by igniting appropriate signal pathways and gradually heating the entire cell and the tumor.

The thermal processes provide optimal conditions by increasing the reaction rate of the chemical reactions ignited by the nonthermal effects [46]. Due to its enormously high fixed polarization, the cell membrane rectifies the periodically changing electric field (≈ 107 V/m) Figure 6(A). [61]. The applied electric power dominantly functions nonthermally [49]. Silico models could approach the best carrier frequency chosen, 13.56 MHz, in the overlapping region of β and δ frequency dispersion, [33], using membrane charging, orientation of transmembrane proteins, dipolar mechanisms, and tumor selection. The chosen modulation was taken from the $\alpha\alpha$ dispersion guiding the electrochemical effects and active membrane processes, including the excitation of transmembrane proteins [3] [15], Figure 6(B). The applied modulated RF triggers apoptotic pathways [53], and the nonthermal antiproliferative membrane effects induce ion fluxes (especially of Ca^{2+}) and/or resonances causing membrane depolarization [33].

Nonthermal activity makes structural changes that affect the intracellular polymerization of cytoskeleton filaments [62] [63]. The fluctuations are also essential in the electromagnetic interaction, showing thermal and electric noise limitation in the TME-connected membrane [64] [65]. The molecular models concentrate on the membrane effects, showing the thermal and nonthermal results. The same heat conditions force the same processes in the cytosol ER and other cellular organelles. The heat-sensitive transient receptor potential vanilloid receptor (TRPV) also senses the same temperature for action. The mEHT causes the excess ionic concentration [7], which increases the influx of Ca^{2+} ions from the ECM to the cytosol. The high iCa^{2+} promotes apoptosis in the mitochondria-dependent intrinsic signal pathway [66].

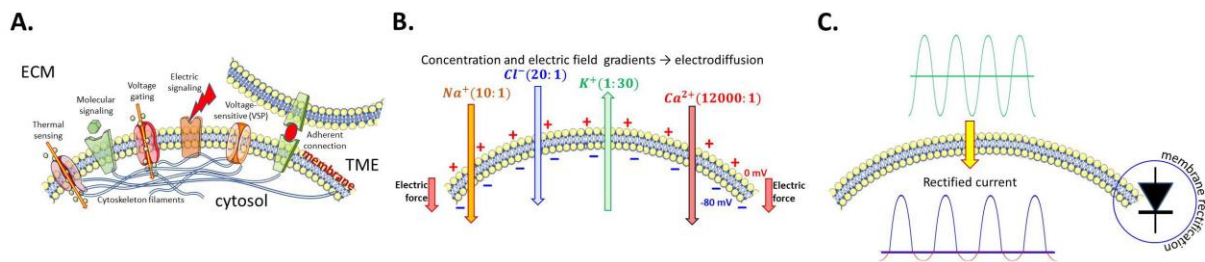


Figure 6. Nonthermal membrane processes. (A.) The various ion channels are sensitive to different ignitions, including thermal and non-thermal effects. (B.) The ion gradients and the field gradient make electrodiffusion, working differently on the various ions (only the most common ionic exchanges are shown). The membrane controls the ion flows similarly to the transistor effect. (C.) The membrane rectifies the electric field, working like a diode.

A remarkable feature is that the thermal and nonthermal effects have similar electrical mechanisms with modulation [50], harmonizing the complex interaction phenomena of the electric field. The nonthermal activity causes structural changes affecting the intracellular polymerization of filaments [2]. The fluctuations also have an essential role in the electromagnetic interaction, showing thermal and electric noise limitation in the TME-connected membrane [64] [65].

4. PHANTOM MEASUREMENTS

Various phantom systems were checked for the thermal effect of mEHT. The publications of phantom research are summarized in Table 2.

Table 2. Phantom measurements in preclinical research.

Material	Method	Result	Conclusion	Ref.
Agar phantom (6 kg, 24 × 24 × 8.5 cm).	mEHT, Ø30 cm applicator, 150 W, 30 min, infrared thermometry.	> 3.5°C temperature increase, absorbed ~24.5 W/kg, with 74% efficacy, bulk absorbs in a cone shape, at the bottom 50%.	The phantom shows high efficacy of bulk heating with satisfactory penetration depth.	[67] [68]
Egg-white in distilled water, cylindrical tank, Ø10 cm, height 25 cm.	mEHT temperature measurement in water and egg white, 75 W, 15 min.	Significant temperature difference, egg white has a 5-times increase, which agrees with the simulation.	The heating selection in the distilled water + egg white heterogenic arrangement is proven.	[69]
Chopped porcine meat (mixed muscle), cylindrical tank, Ø10 cm, height 25cm.	mEHT, temperature measurement in depth by 5 cm, 75 W, 1 h, Ø10 cm applicator.	The temperature rises from 23°C to 44°C on the top and to 40°C at the bottom after 1 h. The temperature decreases linearly with the depth.	The heating efficacy and the reached temperature were appropriately high, as expected in clinical applications.	[69]
Chopped porcine meat (mixed muscle), cylindrical tank, Ø10 cm, height 31 cm.	mEHT, temperature measurement in depth by 5 cm, 100 W, 1 h, Ø10 cm applicator.	The temperature rises from 27°C to 55°C on the top and 45°C on the bottom. There was a hump of temperature in 10 cm depth.	The heating efficacy is high, and the method could reach high temperatures.	[70]

Egg white, pork liver, and red caviar in distilled water.	mEHT, protein denaturalization, and coagulation measurement in the samples. Ø20 cm applicator.	The liver showed inside denaturalization, egg white coagulated, and caviar was cooked inside, while the water temperature increased only ~3°C.	The selection of energy absorption is proven in these phantom arrangements. [70]
Chopped pork muscle + fat meat phantom, 6.6 - 7.2 kg in Ø20 cm and 20 cm high plastic cylinder. The impedance of the sample was 23.9 + i76.8 Ω.	mEHT 150 W, 1 h (~540 kJ), thermally isolated setup, Ø20 cm applicator.	Temperature rises from 22.5°C to 38°C (ΔT~15.5°C) at the top and 35.5°C (ΔT~13°C) at the bottom.	The temperature growth shows appropriate conditions in this model for clinical use. [71]
Chopped pork muscle + fat meat phantom, covered with paper soaked in saline, mimicking the patient's sweating.	mEHT 50, 100, 150 W, 1 h, thermally isolated setup, Ø20 cm applicator.	The shape of the temperature rise changes. The maxima of ΔT are 2°C at 500 s, 8°C at 150s, and 15.5°C at 1600 s for 50, 100, and 150 W power, respectively.	The patient's perspiration robustly raises the surface temperature in this phantom model and could reach thermal toxicity in the skin. [71]
Pocrine ribs with skin, muscle, and connective tissue. The liver is placed in the middle of the phantom, forming a "sandwich" arrangement.	mEHT Ø20 cm applicator, 100 W, 15 - 26 min. The ready phantom was ~10 cm thick. A metallic implant (hyp replacement) was also tried inside the "sandwich".	The temperature rises at 26 minutes on the skin, at 16.6°C - 17.3°C, ribs 9.5°C - 14.3°C, liver surface 7.9°C - 8.5°C, and liver inside 6.2°C - 6.6°C. Metallic implant 9.5°C. Temperature growth was ~1.2°C by 5 min in all samples but stabilized at 38.2°C, 40.1°C, and 43.2°C in 0.32, 0.06, and 0.03 L/min/kg perfusion, respectively.	The rise in the temperature of the liver in the phantom was enough for clinical requirements. The skin, however, would have thermal toxicity. (No blood circulation.) [72]
Porcine liver using arterial hepatica to model blood perfusion.	mEHT Ø20 cm applicator, 100 W, 60 min. 0.03, 0.06, and 0.32 L/min/kg perfusions were modeled.		The mEHT method looks satisfactory for heating tissues with blood perfusion. Thermal toxicity in the skin is less probable. [72]
Multilayer "sandwich" structure of pork with layers of skin, adipose tissue, muscle, liver, and adipose tissue skin in Ø20 cm and 20 cm high plastic cylinder.	mEHT, Ø20 cm applicator, 100 W, 1.5 h.	The maximum temperature was reached after 65 minutes, at 32.5°C in healthy and 37°C in tumorous liver.	The mEHT may select between the phantom structure's healthy and tumorous liver tissues. [67]
Spherical model for necrotic tumor. Outside deionized water, inside egg white and 0.9% NaCl saline is embedded in the egg white to model the necrosis.	mEHT, Ø20 cm applicator, 100 W, 20 min. The experiment was done on the water mattress of the treatment bed of the EHY2000+ device.	From 23°C, the maximal temperatures are 53°C for egg white, 43.5°C for saline, 34.5°C for deionized water around, and 28°C for the treatment bed underneath.	The selection was shown with this multilayer spherical model. [73]

The conventional thermal impact is measured with an agar phantom [68]. The phantom had a 6 kg weight (24 × 24 × 8.5 cm size) treated with 150 W for 30 min, treated with an EHY2000+ device using a 30 cm diameter applicator Figure 7. The measurement with infrared thermography shows only the surface temperature at 22°C room temperature, which is considerably lower than in the bulk material. The observed shape of the heat distribution was a truncated cone, and the temperature was increased by ~4°C, in good linear approximation (R2 = 0.9691).

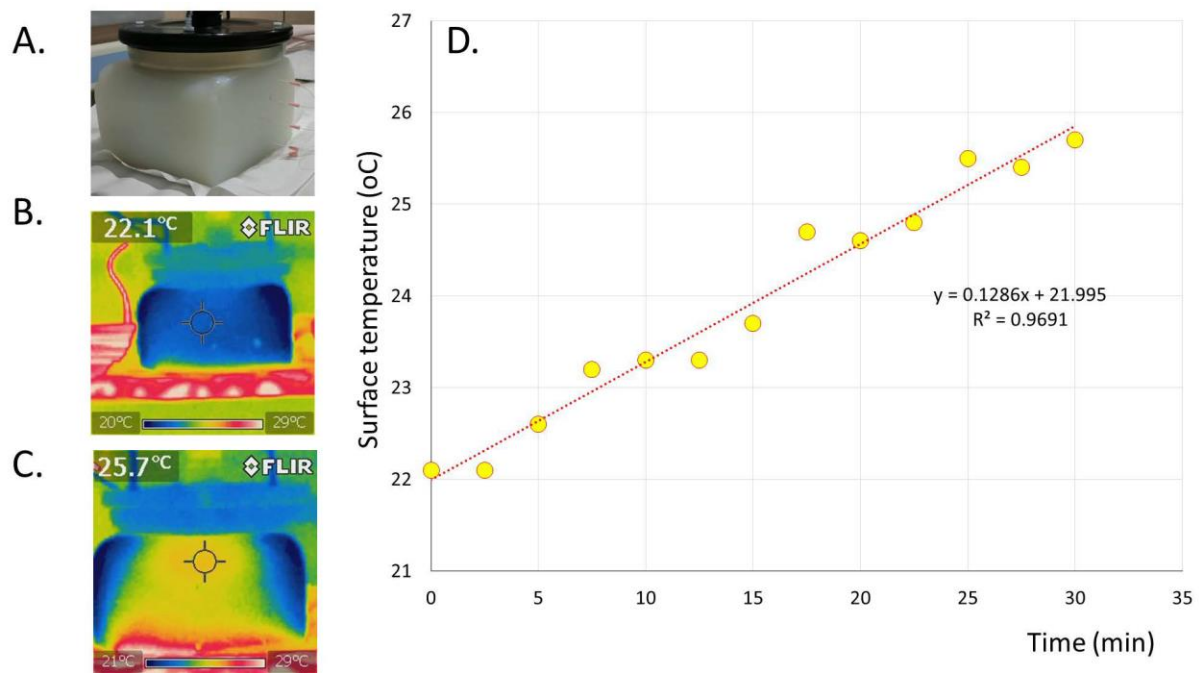


Figure 7. The agar phantom measurement. (A.) The temperature distribution is measured by thermometry. (B.) Linear dependence of the surface temperature from the time.

For more realistic experiments, chopped port meat (a muscle and fat mixture) was placed in a long cylindrical plastic holder. Three different setups were measured at other times (Figure 8). The dynamic linear temperature development is clearly shown in all experiments [69]–[71].

Phantoms were used to study the method's energy focus. Various actual tissues (pieces of liver, egg white, caviar) were placed in distilled water in the cylindrical holder [74]. A more realistic phantom was developed with a liver embedded in pork ribs (Figure 9 [72]). The temperature measurement of various phantom parts proved the satisfactory temperature increase. The same model was used for a larger metallic implant in the treating volume. The inserted hip joint was not heated extremely, proving the safety of the implants in the targeted volume. The effect of blood flow was also modeled with this experimental setup, pumping room-temperature electrolytes into the arteria hepatica. Without blood perfusion, the temperature changes rapidly, while higher perfusions moderate the temperature change and reach the thermal balance sooner.

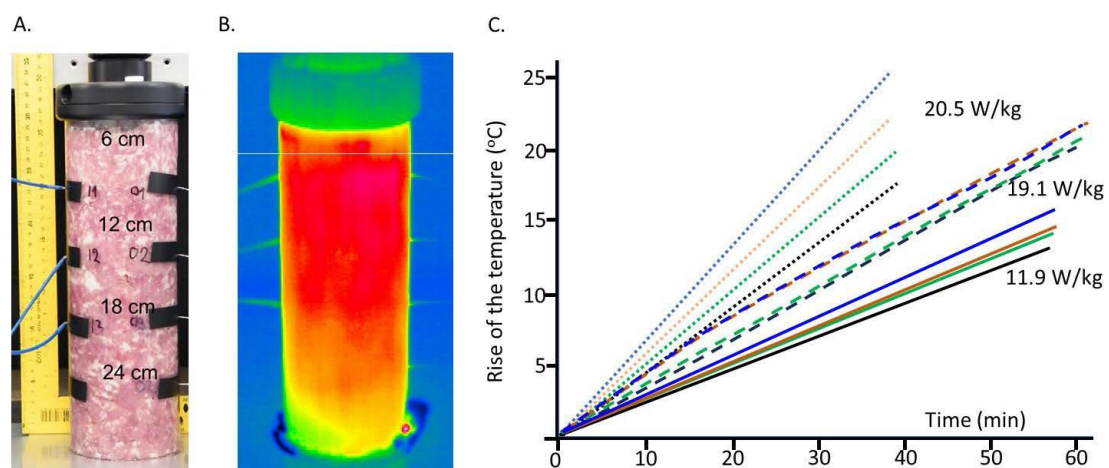


Figure 8. The Cylindric chopped meat phantom experiments. (A.) Meat phantom setup from [69]. (B.) The infrared thermography of the phantom after 40 min treatment. (C.) The various phantom experiments showed a linear temperature development, in which the slope depends on the setup. The experiment with SAR = 20.5 W/kg was made in a cylinder 31 cm in length and 10 cm in diameter, and 100 W power (40 min) was used with a 10 cm diameter applicator [70]. The experiment with SAR = 19.1 W/kg was done in a cylinder 30 cm in length and 10 cm in diameter, and 75 W (60 min) power was used with a 10 cm diameter applicator [69]. The experiment with SAR = 11.9 W/kg was done in a cylinder 20 cm in length and 20 cm in diameter, and 150 W (60 min) power was used with a 20 cm diameter applicator [71]. The results correspond well with the in silico simulations [69].

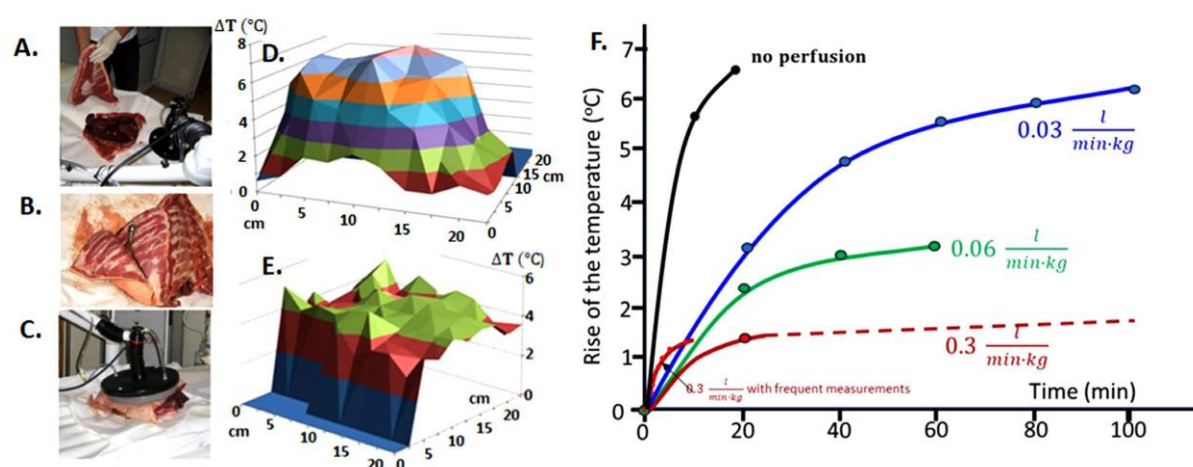


Figure 9. The pork rib phantom experiment (100 W). (A.) Pork liver “embedded” in the pork rib. (B.) A metallic hip replacement placer in the rib of pork. The most significant temperature of the inserted metallic implant after 1 h treatment was 9.5°C. (C.) The treatment of the “sandwich” structures with the EHY2000 device. (D.) The temperature rises in the subcutis layer at 20 min 100W. The most considerable temperature in the skin after 1 h treatment ranges from 16.6°C – 17.3°C. (E.) The temperature rises in the embedded liver after 20 min 100 W. The most significant temperature in the liver after 1 h treatment ranges from 6.2°C – 8.5°C. (F.) The phantom simulation of the blood perfusion with the liver phantom forcing room-temperature electrolytes through the arteria hepatica. The measured discrete points (20-minute intervals) are connected to guide the eye. The large perfusion case was measured at a higher frequency (1 min intervals) at the start of the temperature rise, showing a more precise quasi-isothermal start.

The phantom measurements describe the processes only partly. They have no dynamic feedback. Primarily, the physiologically regulated blood perfusion is missing. In the last experiment (Figure 9), the perfusion was modeled, but no feedback regulation was possible.

5. IN VITRO MEASUREMENTS

Modulated electro-hyperthermia (mEHT) was studied in vitro. The mEHT is a hyperthermia, which targets tissue’s natural electric and thermal heterogeneities [74]. It recognizes the cancer cells selectively and destroys them [19]. The electric field selects the tumor, and the low-frequency amplitude modulation polarizes and excites the transmembrane proteins of the malignant cells [16]. The dominant absorption of the energy by the microscopic clusters of the membrane rafts

acts like nanoparticle heating [4]. Exciting the membrane produces various apoptotic signals. The in vitro experiment had a glass cuvette with distilled water, and the condenser applicators were tightly attached to the cuvette walls. The investigated cell suspension was placed in a polyethylene plastic bag and immersed in the water Figure 10. The LabEHY (Oncotherm, Budaors, Hungary) provided a well-controlled amplitude-modulated radiofrequency (RF) signal with a 13.56 MHz frequency. The temperature was controlled with specialized thermometers immune to RF radiation. The experimental conditions are discussed in detail in every referred article.

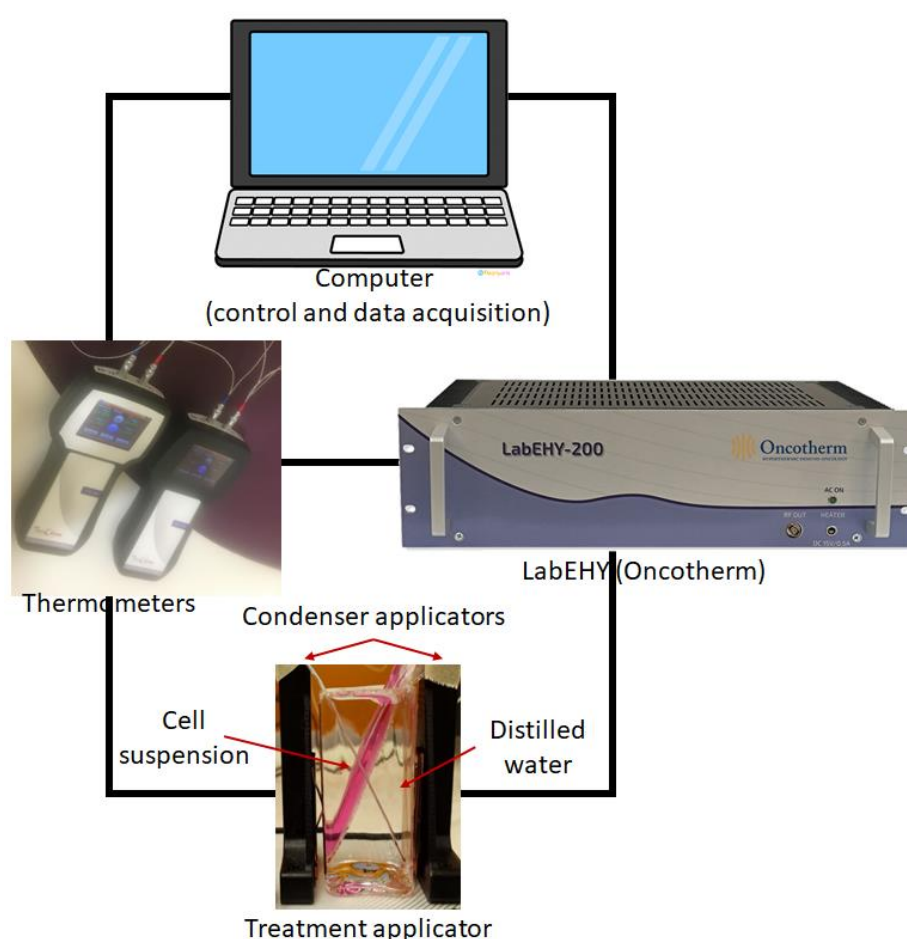


Figure 10. The experimental setup of the in -vitro application.

The in vitro research covers multiple cell lines and various methods to evaluate the results. The most frequent methods are Western blot, immunohistochemistry, flow cytometry, MTT assay, TUNEL, and clonogenic assay. The various results are shown in Table 3.

Table 3. In vitro preclinical research.

Tumor cells	Method	Result	Conclusion	Ref.
mEHT treated coculture of human fibroblast and, HCK and HaCat, A431cells.	The same treatment was given to the fibroblast together with cell variants with different proliferation forces.	The mEHT treatment did destroy the malignant A431 cells, but the healthy and nonmalignant cells remained unchanged.	The mEHT treatment is selective for malignant A431 cells <i>in vitro</i> .	[75]

mEHT treated Huh7 and HepG2 cell lines.	mEHT (3x), 42°C, 60 min, Clonogenic analysis, Western blot, RNA sequencing.	Significant apoptosis by an increase of tumor suppressor SEPT4 with the restoration of p21.	Tumor suppressor SEPT4 mediates the massive apoptosis in HCC cells. [76]
mEHT treated HepG2 cell line.	mEHT 42°C, 30min. Comparison of cHT and wHT at 42°C. Measured with Annexin V positive cells, SubG1 ratio, Western blot.	mEHT had significantly higher apoptosis than other methods. wHT produced a high apoptotic rate as mEHT (42°C) at 45.5°C. mEHT significantly increased the caspase 3, 8, 9, and the extracellular HSP70. Calreticulin and E-cadherin also increased by mEHT.	mEHT causes significantly higher apoptosis than other methods by the selective energy deposit. [77]
MCF7, WiDr and U87MG cell-lines treated with mEHT.	mEHT 42°C, 30 min. cHT and wHT at 42°C. Measured with Annexin V positive cells.	The apoptotic rate was significantly higher than wHT and cHT at the same 42°C temperature	The heterogeneous mEHT is superior to homogeneous heating intentions. [77]
CT26 murine colorectal cancer cell line treated with mEHT.	mEHT 42°C, 30 min, 8 - 9 W comparison with wHT same temperature and time. Annexin V/PI, flow cytometry, Western blot, ELISA	Significantly higher apoptosis with mEHT than in wHT at the same temperature. HSP70 was released from the cells. mEHT induced a greater HSP response than wHT did.	mEHT is a significantly better apoptosis inducer than homogeneous wHT heating. [78]
OVCAR-3, SK-OV-3, HeLa and SNU-17 cells treated with mEHT.	Apoptotic markers such as cleaved are measured. FACS and Western Blot were used.	Significant apoptosis was measured in all cell lines. p-P38, c-Cas-3, and PARP were increased.	mEHT treatment is sufficient for the degradation of tumor cell lines. [79]
CT26 murine colorectal cancer cell line treated with mEHT.	mEHT 42°C, 30 min, 8 - 9 W reacted injected DC. MHCII, CD80, and CD11c DC surface markers are measured with FACS analysis for matured and immature DC cells.	mEHT changed the DC surface markers in both the matured and unmatured cells. The fluorescent intensity of MHSII and CD80 was significantly higher in mature DC than in immature counterparts.	mEHT changes the DC surface markers in both DC states but is significantly higher in mature states. [78]
Aggressive and radioresistant 9L rat gliosarcoma and MCF-7 and MDCK cell lines treated with mEHT.	Clonogenic assay for mEHT treated 9L cell line combined with megavolt radiation: propidium iodide staining, 10 MV, 5 Gy.	mEHT selectively attacked the L9 cells, but those were shortly repaired. The proliferation arrested cancerous MCF-7, but the non-cancerous MDCK remained unchanged. mEHT and the RT synergetically induced cell death, reducing normalized survival.	Combining mEHT with megavolt irradiation could cause cell death for the radioresistant L9 cells. [80]
Panc1, a KRAS and TP53 mutant, radioresistant pancreas ductal adenocarcinoma cell line treated with mEHT.	mEHT 20 - 25 W 5min until 42°C, keep with 7 - 8 W for 60 min. Cell viability, apoptosis by Annexin V/PI, immunohistochemistry, flow cytometry, clonogenic assay, and Western blot were applied.	mEHT made significant apoptosis; mono and combined treatments affected the tumor progenitor/stem cell populations. Accumulation of γ H2AX indicates DNA double-strand breaks and upregulation of the cyclin-dependent kinase inhibitor p21 ^{waf1} .	mEHT treatment can contribute to tumor growth inhibition and apoptosis induction and resolve radioresistance of Panc1 cells. [81]

Panc1 and Capan1 cell line, ALDH+ cancer stem cell (CSC) fraction.	mEHT 60 min + 2 Gy radiation (137 Cs source) (RT), Annexin V/7-AAD staining, immunohistochemistry and flow cytometry were applied.	mEHT + RT combined therapy caused significantly more apoptotic cell death in both cell lines than the single modalities alone. The CSC fraction was reduced by mEHT involvement, but RT did not change it.	The leading factor of mEHT application alone or combined is the induction of γ H2AX and significant reduction of CSC.	[81]
HT29 and SW480 human colon cancer cell lines were treated with mEHT.	mEHT and wHT are compared with proliferation and clonogenicity.	mEHT significantly reduced the proliferation and clonogenicity in both cell lines compared to wHT.	mEHT creates significant nonthermal effects in addition to the thermal.	[61]
CT26 mice colorectal cancer cell line + reservatol (Res) and nanosized curcumin (Cur) combined with mEHT.	mEHT and wHT comparison, 42C 30 min, 10-12 W, Annexin V-FITC, Western blot, Immunofluorescent analysis FACS.	Cell viability decreased by Res and Cur. Cell-cycle arrest by Res + Cur, Apoptosis, and immunogenic cell death were detected, and CRT development was significantly higher when mEHT was combined with Cur + Res.	The complementary treatment of mEHT with Cur and Res shows promising potential for clinical applications against malignancies.	[82]
C26 mouse colorectal cancer cell line + doxorubicin (Dox) treated by mEHT.	mEHT 42°C, 60 min (2x), 1 μ mol/L Dox. immunohistochemistry and qPCR, as well as time-course follow-up, were applied.	mEHT with Dox made significant CRT and HSP70 development, reduced antiapoptotic and increased proapoptotic molecules with p21 ^{waf1} .	mEHT activates both caspase-dependent apoptosis and p21 ^{waf1} -mediated growth arrest pathways by the p53 protein.	[83]
Mouse colorectal adenocarcinoma C26 Double tumor where only one is treated with mEHT.	mEHT 42°C, 30 min (2x) and 60 min (2x). Measured cellular morphology, apoptosis, immunochemistry, Western blot, flow cytometry.	Significant cell damage compared to the untreated tumor, significant CRT and HSP70 development, inactivation of pRAF, and activation of pERK1/2.	mEHT causes significant apoptosis and DAMPs, so it can potentially induce immunogenic cell death.	[84]
A549 cell line treated with mEHT.	mEHT and wHT comparison in heating phases (25°C→37°C, 37°C-42°C) and in thermal equilibrium (42°C 30 min, 60 min) The power pulsing was also studied.	It is proven that mEHT produces a significantly higher apoptosis rate than wHT. The main apoptotic phase is connected to the heating process's growing temperature (dT/dt).	The time derivative of temperature is the primary addition to apoptosis with mEHT. It may be used for the pulsed strategy of the mEHT treatment.	[85]
U87-MG and A172 cells treated by mEHT.	mEHT (42°C, 60 min) three times every 2nd day tested for growth inhibition using MTT colorimetric, FACS, and microscopic analysis.	The growth inhibitory effects were observed. The colony formation was significantly suppressed, and apoptosis was induced in both cell lines. Gene expression of RNA 34 commonly deregulated genes is identified.	mEHT induced the inhibition of cell proliferation and clonogenicity, as well as significantly promoting apoptosis.	[86]
U937 cell line treated with mEHT.	mEHT, 42°C, 30 min. Flow cytometry, Western blot, microarray gene expression, comparison with wHT, gene map, IPA.	Different heat treatments have various responses. mEHT induced significant apoptotic cell death, while wHT had significantly less. mEHT upregulates a whole cluster of genes. The Fas, c-Jun N-terminal kinases (JNK), and ERK signaling pathways were dominant to induce apoptotic cell death in mEHT, whereas the cell-protective mechanisms dominated in the case of conventional heating.	We measured the different responses of wHT and mEHT heating. mEHT activates death-related signal pathways, which wHT does not.	[87]

U937 cell line treated with mEHT.	Compare mEHT to wHT in 39°C - 46°C interval for apoptosis, measured by flow cytometry Annexin V/PI.	The apoptotic rate for mEHT at 40°C was similar to that of wHT at 44°C. However, the high difference remained for 41°C at mEHT measurements.	The mEHT makes the apoptotic rate equivalent to wHT at 4°C lower temperature, assuming that the membrane rafts have such an exceptionally high temperature. [88]
HepG2, A549, U87MG, and CT26 cell lines and liposomal Dox uptake were treated with mEHT.	mEHT 42°C, 30 min, 10 - 12 W (3x). Flow cytometry and immunofluorescence measured the Dox release. Substrate assay and Westre blot were used for the study.	mEHT alone suppressed cell viability, and together with liposomal Dox, its drop was significant. The drug release was the highest, with mEHT 42°C. Uptake of Dox with mEHT was higher than with wHT.	The mEHT-induced particle uptake was by macropinocytosis. Dox + mEHT arrests the proliferation. mEHT-enhanced uptake of Dox may amplify the therapeutic effect. [89]
HepG2 and various shapes and sizes of gold nanoparticles were treated with mEHT.	mEHT 15 W, 10 min. Sphere-like, urchin-like, and rod-like gold nanoparticles. Measured relative cell viability and apoptosis rate.	The mEHT effect was suppressed by the addition of gold nanoparticles. The heat generation with gold nanoparticles was negligible.	The nanoparticle addition worsens the cell-killing. The incoming energy is shared between the membrane rafts and nanoparticles. [90]
HT29, SW480, SW620, LoVo, and HCT116 cell lines treated with mEHT.	Modulated (mEHT) and unmodulated (EHT) treatments were compared. Mechanical, thermal, and electric parameters were measured. A comparison with wHT was also done.	Temperature was significantly higher in wHT than in mEHT and EHT, but the apoptosis was substantially higher in mEHT and EHT. The Young moduli differences at 41°C could vary the resonance frequencies.	Resonance frequencies can be approximated from electricity data (Young moduli), showing agreement with the treatment data. [53]
mEHT for 4T1 and 4T07 cell lines.	mEHT 42°C, 30min temperature rise is 2.3°C/min. Histopathology, RT-PCR, mass spectrometry, and nanostring analysis were done.	One of the most upregulated genes/proteins was the mouse C4 complement C4b. C4b mRNA was measurable by qPCR from <i>in vitro</i> 4T1 cell culture.	mEHT treatment in monotherapy induced a significant upregulation of C4b mRNA 2 h after treatment. HSP70 and C4b relative expressions are proportional to each other. [91]
SCCVII and SAS cell lines.	Comparison of mEHT and wHT in combination with RT. Flow cytometry, clonogenic assay, survival time.	Apoptosis and autophagy are significantly increased with combined RT + mEHT treatment and the thermal component increases the effect.	The increased apoptosis and survival time indicate the excellence of the mEHT + RT treatment. [92]
A549 and NCI-H1299 lung cancer cell lines.	Measurement of radiosensitivity change by addition of mEHT to RT.	Measured α and β values of the linear-quadratic equations of cell survival curves show more than doubling the sensitivity.	The mEHT addition to RT significantly increases the cell sensitivity to radiation. [93]

The cellular selection was shown in coculture experiments, [75] [94]. Co-culture with normal human skin fibroblasts as a model of a squamous carcinoma growing within connective tissue cells (100.000/ml) were exposed to mEHT, incubated for 24 h at 37°C, fixed, and stained with crystal violet. The healthy human keratinocytes (HCK) were unaffected by the mEHT treatment, while the highly proliferating but non-malignant (HaCat) cells had a low distortion rate among the unaffected healthy fibroblasts, and the aggressively malignant A431 cells in the co-culture with healthy fibroblasts show a highly selective distortion of malignant cells, while the fibroblasts were not hurt

Figure 11.

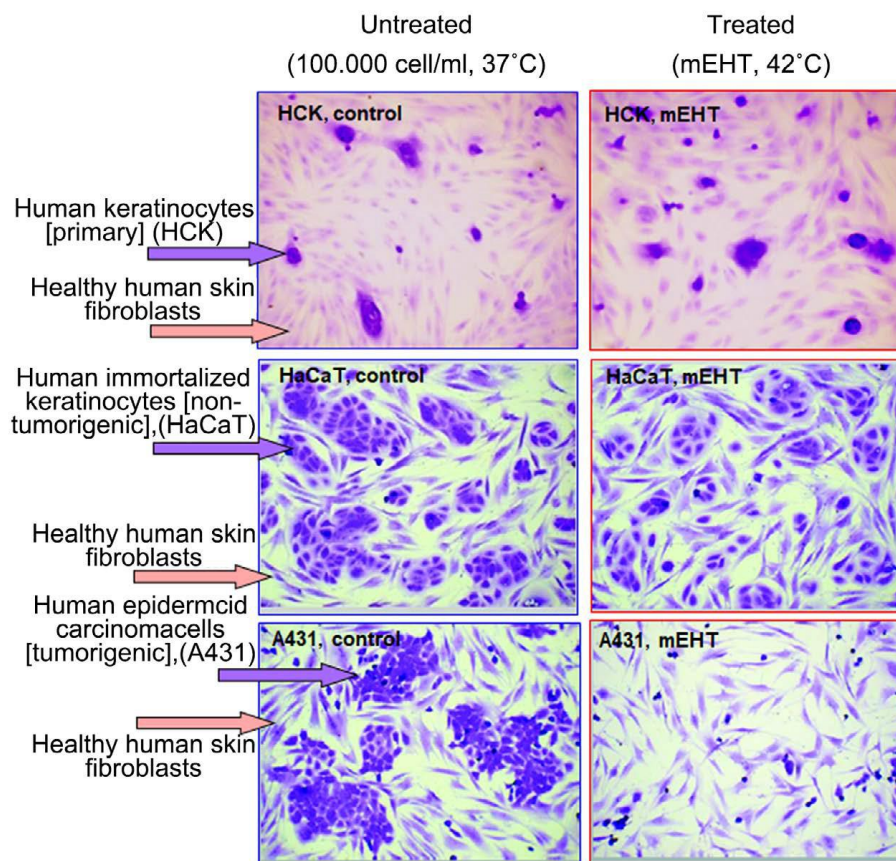


Figure 11. Demonstration of the cellular selection of mEHT treatment (42°C, 30 min) in co-culture of human fibroblasts and healthy, proliferating but not tumor genetic and aggressively malignant keratinocytes compared to the untreated control.

The thermal effects could be calibrated by in vitro experiments. The reference calibration uses the U937 human lymphoma cell line [87] [88], and the HT29 and A431 [13] cell lines. The effect has given a possibility to make a reference calibration of mEHT compared to wHT on HepG2 cells shown at ~5°C [77], while in the U937 cell line [88], it shows a > 3°C shift to the advantage of mEHT over wHT (Figure 12). It is supposed that the difference indicates a 3 + °C higher temperature of rafts than of the TME. The gain of tumor destruction at 42°C is ≈ 4.9 fold. Measurements show the mEHT induces apoptosis targeting the rafts, they have a higher temperature than the average medium indicates, and the temperature of the selected cells (T_{cell}) is well over the average ($T_{cell} \gg T_{mEHT}$).

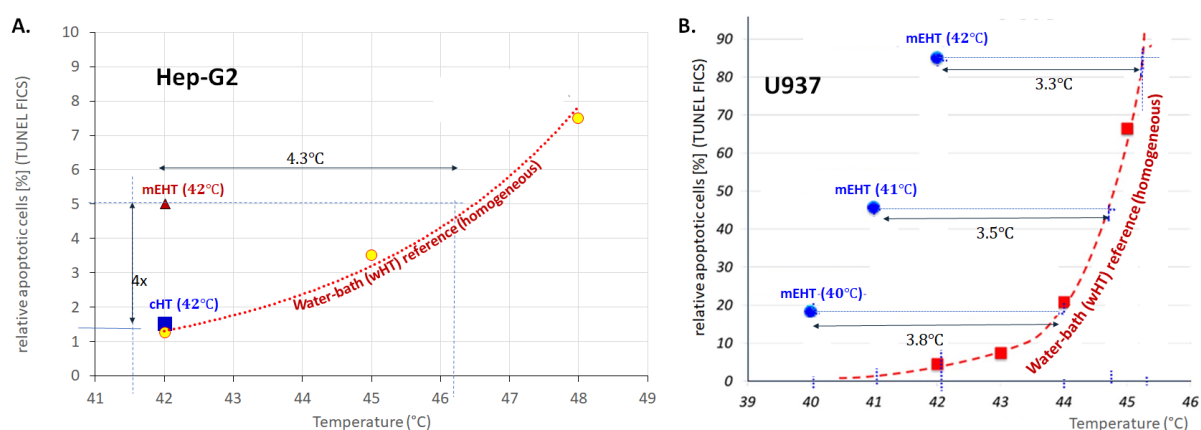


Figure 12. The calibration of the thermal factor by homogeneous conventional wHT. (A.) The wHT calibrates the apoptosis of HepG2 cells by thermal effect (temperature only). The measured mEHT causes effective apoptosis at 42°C. According to the calibration, this corresponds to the 47°C in the calibration curve with the same HepG2 cell line [77]. (B.) Another calibration measurement with the U937 cell line. The mEHT shows a > 3°C temperature difference in apoptotic efficacy at all measured points [88].

Maintaining the temperature compensates for the energy losses, so it needs less dose. The unchanged temperature with lower current density produces significantly less apoptosis as the active heating period raises the temperature [85]; Figure 13. In this way, the apoptotic cellular degradation could be used for dosing during the active heating period. The experiment reveals that the applied power plays a decisive role in the apoptotic processes.

The absorbed power has thermal and nonthermal effects, which we may divide by the high power application but remove the absorbed thermal component by cooling the system. So, the large electric current density (j) makes the nonthermal action effective. Measurements on the U937 cell line prove this concept [95]. The concentration of apoptotic cells grows linearly with the current density j of mEHT; Figure 14. The standard mEHT treatment was performed at 41°C, with a standard current of 2.8A, as shown in Figure 12(B). The current was increased to 3.8A and 4.5A, while intensive cooling suppressed the temperature of the target to 36°C. The control is a sham experiment that fits a linear line with larger-than-standard currents. The heat effect of the standard treatment could be approximated from this experiment, which is under 10% of apoptotic cells, as shown in Figure 12(B). wHT treatment. Approximately 36% higher current (1.36 times more current) as standard at 41°C needs to have the same apoptosis in the cooled sample to 36°C. This gives a value of ~37% of the absorbed energy being nonthermal. Further, 18.5% more current causes 33% more apoptosis nonthermally. The nonthermal impact on HT29 and SW480 human colorectal cancer cell lines shows a significant change in the ionic fluxes. Also, the mEHT nonthermal effect has doubled the antiproliferative and anticlonogenic activity compared to the purely thermal wHT at the same 42°C temperature [61].

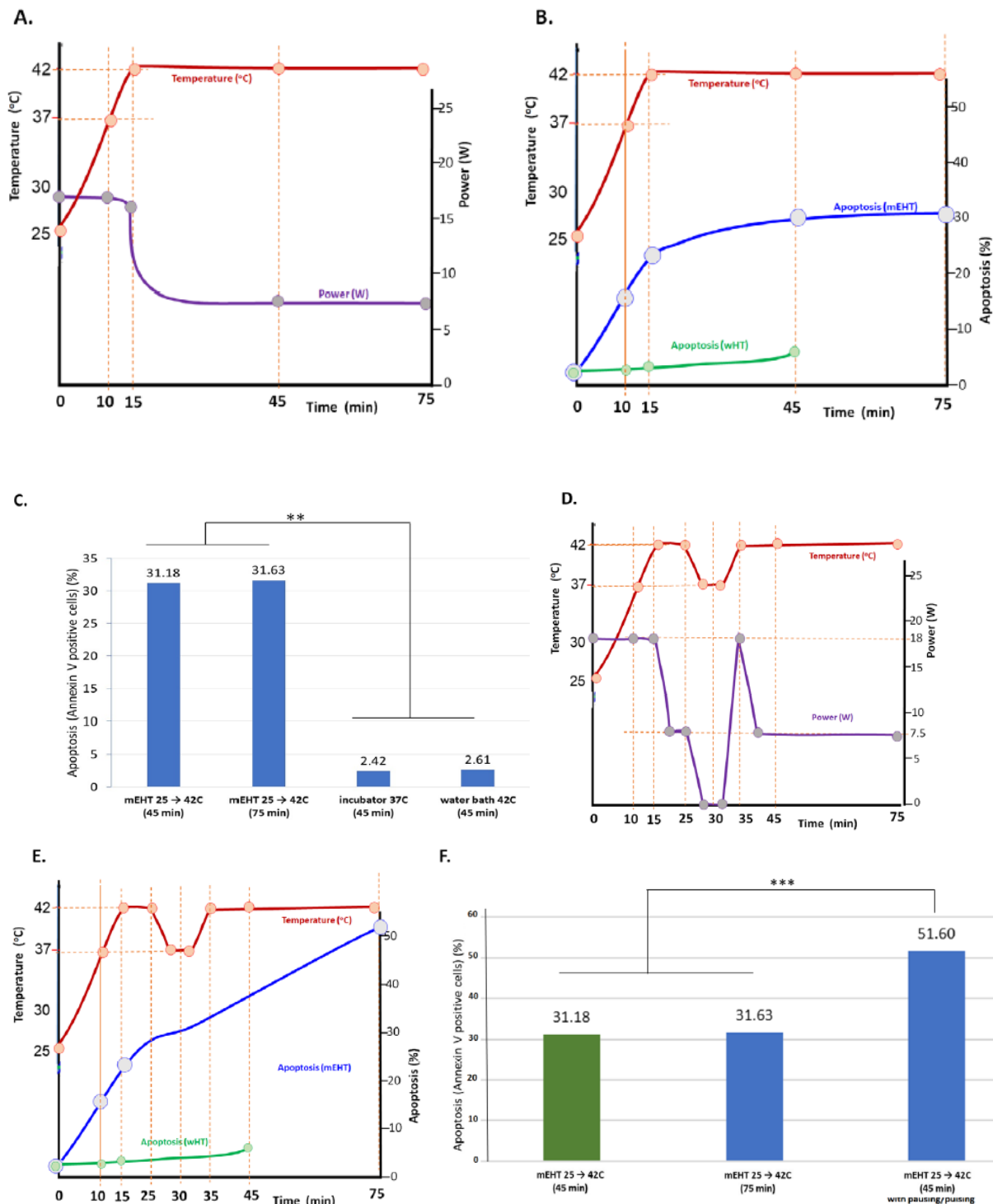


Figure 13. The effect of heating and maintaining the temperature on apoptosis [85]. At the same temperature, the mEHT had significantly more apoptotic cells than the wHT. (A.) The temperature develops over time and with applied power. (B.) The apoptosis saturated when the temperature became constant during the treatment's maintenance period. The apoptosis rate by mEHT is much higher than that of wHT. The slope of temperature rise and the corresponding more extensive power causes most of the apoptotic processes. (C.) The apoptotic cell death is significantly higher with mEHT than with wHT or incubator. (D.) The thermal equilibrium was interrupted by a cooling to 37°C by a power pulsing. (E.) The apoptosis drastically increased by the power pulse. (F.) The pulsed mEHT provides a very significant increase in apoptosis.

The percentage of the apoptosis induced by mEHT grows by increasing current density, which participates in both fundamental processes of this method: in the thermal and nonthermal action components. The thermal effects ensure the conditions for optimal nonterminal (excitation) processes and the rates of chemical reactions (mostly enzymatic assistance) afterward. The current density j appears as an ultimate dose of mEHT, as shown in Figure 14. We may regard the current density as a dose of the treatment, having the same role in mEHT as the ionizing isodose in RT. The qualitative dose equivalence of mEHT with RT defines the harmonizing basis of cellular degradation in two different lung cancer cell lines, A549 and NCI-H1299 [93]. The thermal radiosensitivity parameters were approached by the standard linear-quadratic model. The RT (2 – 8 Gy) + mEHT (42°C, 30 min) increased the linear factor (α) of A549 and NCI-H1299 cell lines from 0.23 Gy to 0.53 Gy and from 0.24 Gy to 0.51 Gy, respectively, which shows the doubling of radiosensitivity with mEHT addition [93].

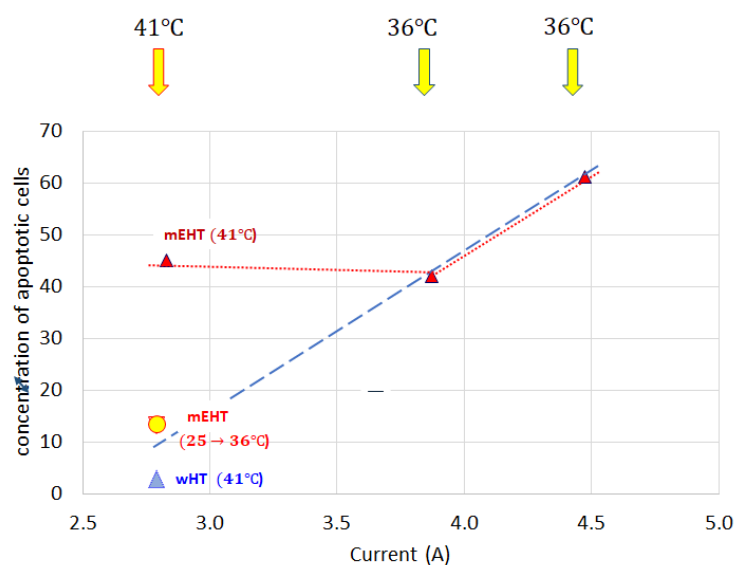


Figure 14. The apoptosis increases with the increase of current density [95]. The higher current density was reached by intensive cooling of the sample, keeping the medium at 36°C, while the standard treatment was at 41°C. (A red dotted line connects the measured data points to guide the eye.) The measured control apoptosis at 36°C fits a line with the cooled higher current on the same 36°C temperature. The apoptosis produced by wHT is less than the 36°C control of mEHT. (The values fit to Figure 12(B))

The gold nanoparticles (NPs) are frequently chosen for selective nanoparticle heating with a 13.56 MHz electric field [96]. This selective energy absorption provides a pure thermal effect for NPs, those thermally absorb the energy and heat their environment. Gold NP suspension was added to increase the thermal impact of mEHT [90]. NPs addition significantly reduced the apoptosis of the HepG2 cell line compared to the mEHT stand-alone treatment (Figure 15). The energy-sharing between the membrane rafts and the NPs probably causes this contradictory effect. The phenomenon supports the proof of the nonthermal energy absorption selection by mEHT. Further noteworthy information is that the intracellular NP addition has significantly more apoptosis inhibition, which depends on the disturbance of the apoptotic signal pathways in the cell.

Comparison of the modulated (mEHT) with the unmodulated (EHT) treatments heating the same experiment setup with 10W power gives further clues of the nonthermal impact of mEHT [53] Figure

16. The power deposition of the medium without cells is significantly lower than in the cancer cell treatments, probably due to the cells' selective and intensive energy absorption. 1 million cells were in 1ml (approximately 0.1% of the volume), and the power deposition increased by ~20%, showing the method's high cellular selectivity. The EHT absorbed significantly more rate (~5%) than the mEHT. The rate difference could be due to the nonthermal processes, which use energy but do not increase the temperature. Consequently, we may approximate the nonthermal energy consumption of 5% of the incident power.

Apoptosis changed significantly in all cell lines compared to wHT, but the mEHT differed significantly only in the HT29 cell line in this experiment. The timing of apoptosis measurement is critical because this process requires a longer time. The measurements were done in vitro a short time after treatment when only early apoptosis could be detected.

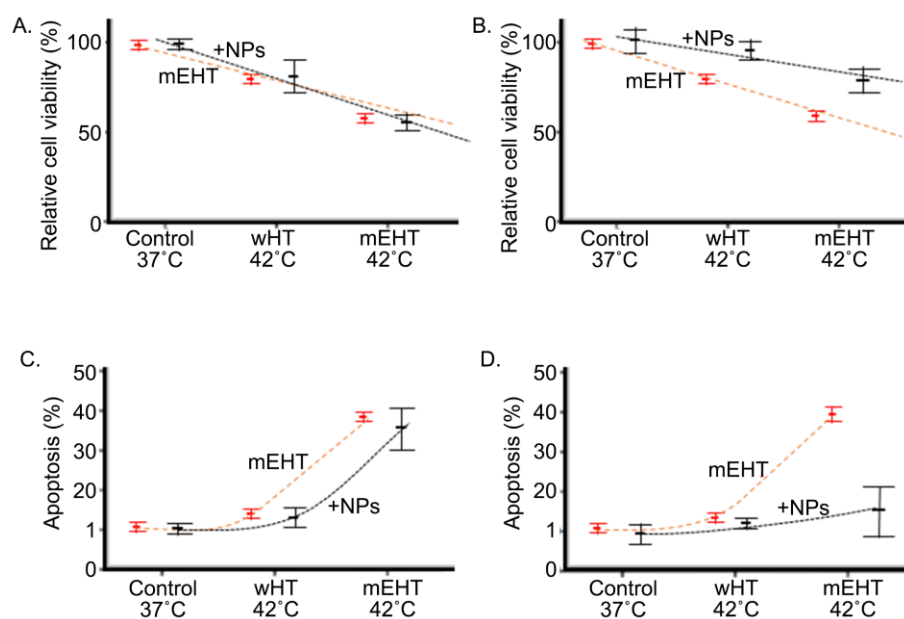


Figure 15. The effect of gold nanoparticles on the wHT and mEHT results at the same 42°C temperature in the HepG2 cell line [90]. The red markers are mEHT stand-alone, and the black ones are the various shapes of NPs. The red dashed and black dotted lines guide the eye, respectively. (A.) The relative cellular viability was measured by MTT assay with extracellular NPs. (B.) The relative cellular viability was measured by MTT assay with intracellular NPs. (C.) The relative apoptosis with extracellular NPs. (D.) The relative apoptosis with intracellular NPs.

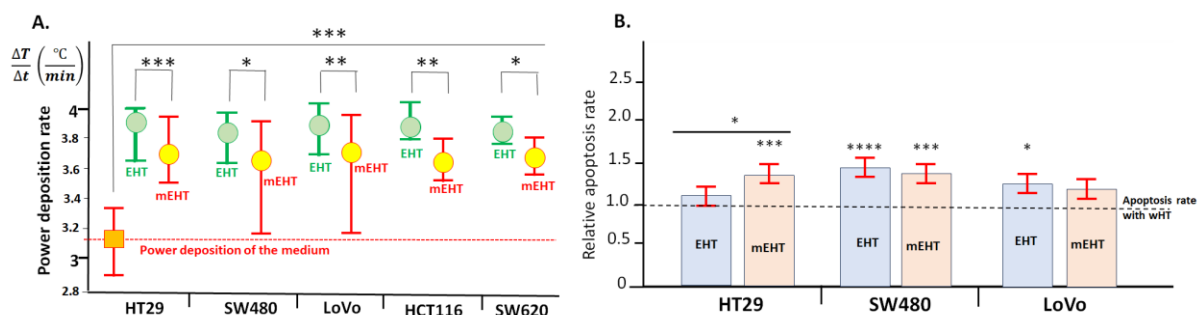


Figure 16. Effect of modulation in vitro for various colorectal cell lines [53]. (A.) The power deposition rate (temperature time derivative) supports the selection and the nonthermal effects.

(B.) the relative apoptotic rate to the control is > 1 in all cell lines. The differences between modulated and nonmodulated treatments are significant only in the HT29 cell line.

The apoptotic rate is significantly higher by mEHT than that caused by wHT. It is a notable observation that the cell distortion does not change significantly between 24 h and 48 h posttreatment measurements, so the direct cell distortion is finished 24 h after the treatment, and the processes turn to such molecular changes, which appear later in the system. Consequently, the standardized experimental protocol used 24 h after treatment investigation with 42°C, 30 min treatment conditions. The comparison of mEHT to wHT and to plane-wave fitted, non-modulated capacitive hyperthermia (cHT) at the same temperature shows significant differences in the cell cycle control after 24 h of the hyperthermia treatments in the HepG2 cell line [77]. Different cell lines showed the dominance of apoptosis of mEHT in comparison with wHT at the same standard treatment conditions Figure 17. mEHT applications focus on induced apoptosis [97] [98].

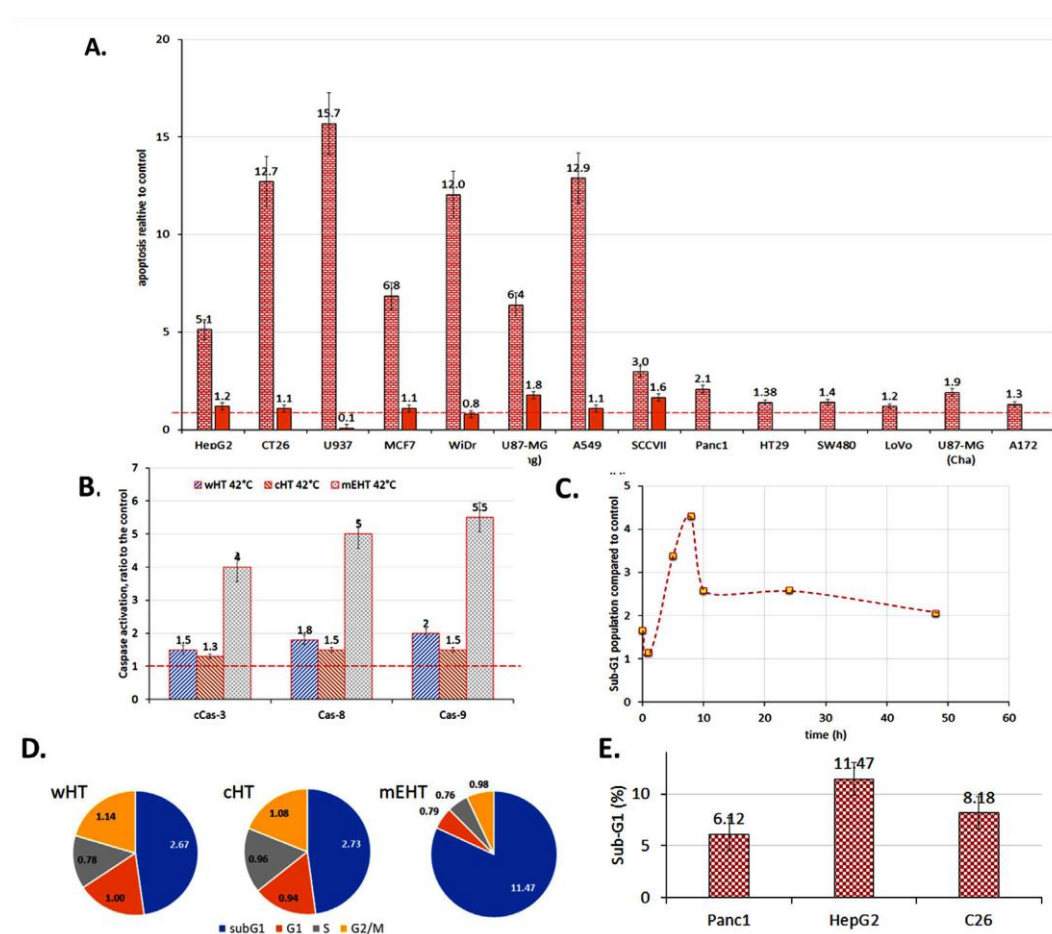


Figure 17. The cell death of various cell lines. (A.) Apoptosis (relative to the control) comparison of wHT and mEHT using the same experimental conditions 42°C 30 min. The results on cell lines were published: HepG2 [77], CT26 [78], U937 [87], MCF7 [77], WiDr [77], U87-MG (Yang) [77], A549 [85], SCCVII [92], Panc1 [81], HT29 [53], SW480 [53], LoVo [53], U87-MG (Cha) [86], A172 [86]. (B.) The cleaved Caspase-3 (cCas-3), the Caspase-8 (Cas-8), and Caspase-9 (Cas-9) show the external and internal pathways of apoptosis and appeared significantly more than in wHT or cHT hyperthermia methods [77]. (C.) The Sub-G1 cell population by time peaks at 8 h where the DNA fragmentation happens in the OVCAR cell line. [79] (D.) The sub-G1 apoptotic character in the cell

cycle is dominant for mEHT treatment [77]. (D.) Comparison of SubG1 measurements Panc1 [81], HepG2 [77], C26 [83]. (E.)

The stand-alone mEHT treatments demonstrate the capability of the method. The experiment proves the superiority of mEHT and presents similarities between wHT and cHT, both of which are devoted to homogeneous mass-heating, while mEHT has heterogeneous selective action. The mEHT method causes caspase-dependent paths through Cas8 (extrinsic way) and Cas9 (mitochondrial, intrinsic way) [77] [99] and independent [100] [101] apoptosis. The detailed molecular investigation shows the apoptosis in mEHT processes Figure 18. Apoptosis regulation-related gene expression at the mRNA level, such as XIAP, BCL-2, and BCL-XL, is suppressed compared to the control. In contrast, the proapoptotic ones (PUMA, BAX, BAK-1) increase, and both groups return to baseline at 9–24 h. A notable factor at the protein level is the arrest of the XIAP effect to block the main path of caspase-dependent apoptosis by the secretion of SMAC/Diablo [102] and Septin4 [76]. The p21 cyclin-dependent kinase inhibitor also increased compared to the control (Figure 18(C)), and it could promote or inhibit apoptosis. Noteworthy, the p21 could be a primary mediator of the p53 cell cycle arrest; its presence expresses the wild p53 tumor-suppressor, which also had been increased in the mEHT process (Figure 18(D)).

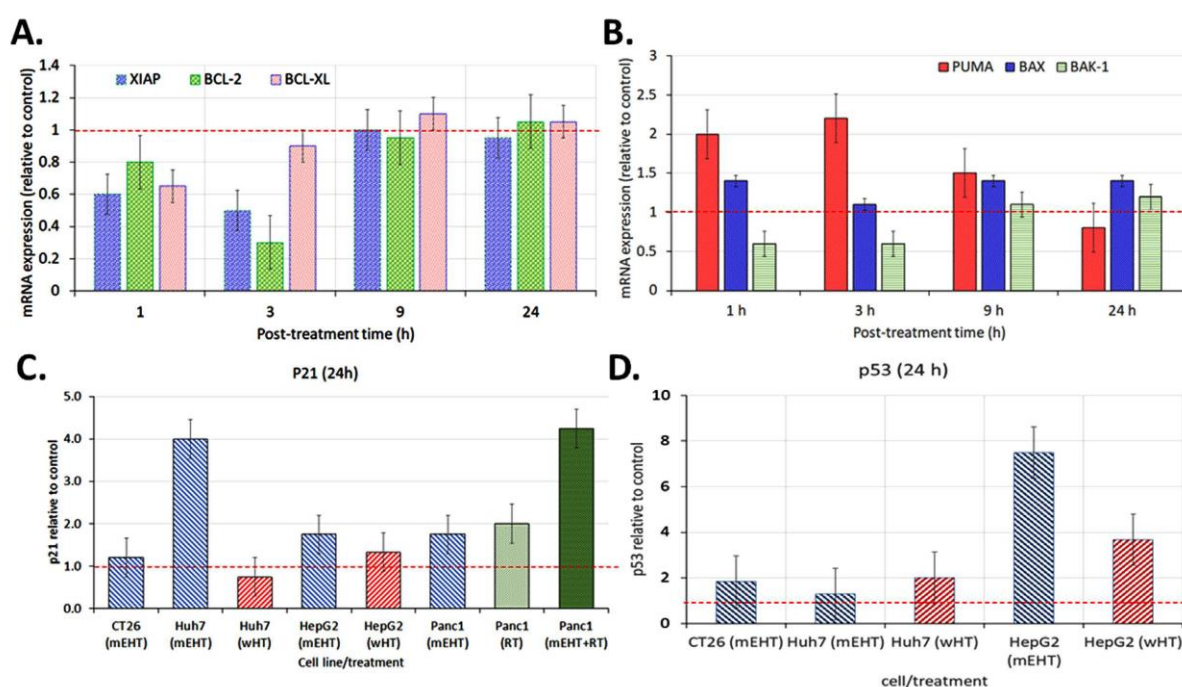
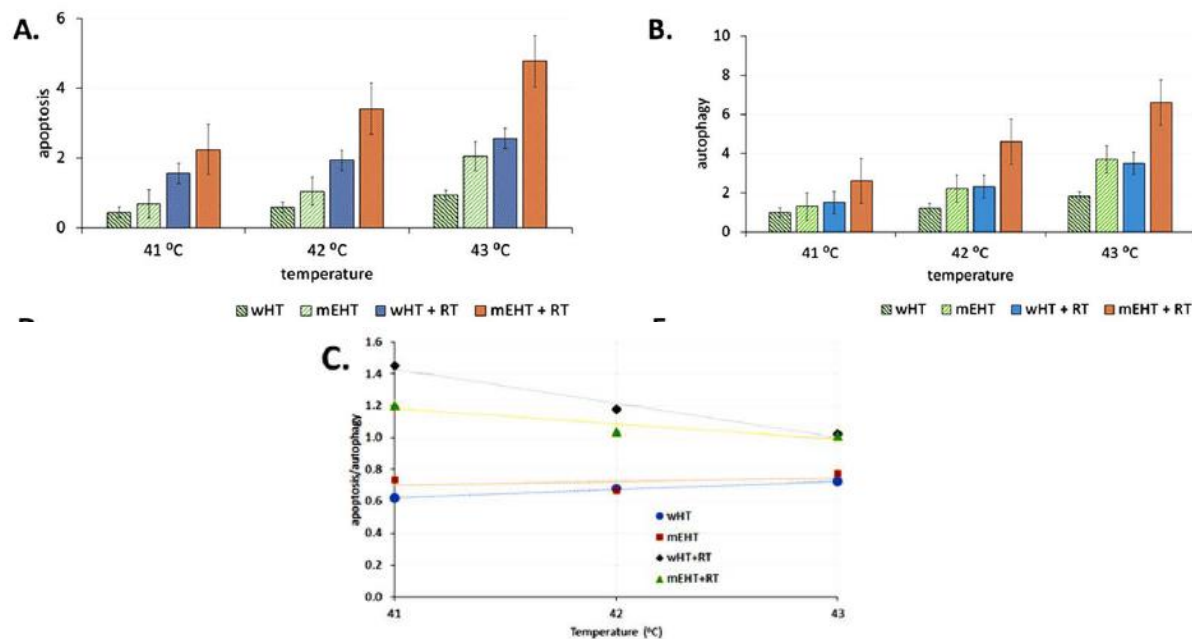


Figure 18. Anti and proapoptotic factors. (A.) Antiapoptotic gene expressions by time development [83]. (B.) Proapoptotic gene expressions by time development [83]. (C.) p21 expression at 24 h posttreatment for different cell lines: CT26 [83], Huh7 [76], HepG2 [76], Panc1 [81]. The wHT, RT, and RT + mEHT experiments are also shown for comparison. (D.) p53 expression at 24 h posttreatment for different cell lines CT26 [83], Huh7 [76], HepG2 [76]. The wHT experiments are also shown for comparison.

The in vitro preclinical complementary treatments also show the superiority of mEHT with add-in complementary applications. mEHT in combination with radiotherapy (RT) significantly improved the apoptotic ratio compared with wHT combined with RT [92] (Figure 19(A)). There was also a substantial increase in autophagy with the mEHT combinations Figure 19(B). Autophagy can act as

a defense mechanism to prevent apoptosis by self-cleaning or recycling the process of reusing cellular components. The mEHT-increased temperature raises the apoptosis/autophagy ratio, which proves that autophagy does not block the apoptotic processes, and excessive autophagy also leads to cell death. (Figure 19(C)). Cell cycle arrest of malignant cells has been demonstrated in the aggressively radioresistant cell line (L9), which mEHT + RT could resensitize [80], and also in complementary mEHT + RT treatment of radio-resistant Panc1 cell line [81] (Figure 19(D)). Caspase-involved extrinsic and intrinsic apoptotic signal pathways accompany the resensitizing [81] [92], (Figure 19(E)). The apoptosis in the function of the phosphorylated H2AX (γ H2AX) serves as a marker of DNA damage, and repair shows peculiarity in the radioresistant Panc1 cell line. The mEHT with the same γ H2AX RT causes more apoptosis than RT, indicating the possibility of different apoptotic pathways. Still, RT damages the DNA strands; mEHT affects apoptotic pathways of the cell, having fewer DNA breaks in the process but more apoptotic outcomes because this death form does not depend on the DNA repair enzymes. Furthermore, the combined mEHT + RT treatment has a significantly higher γ H2AX, indicating aggressive DNA damage in the apoptotic process. Although the apoptosis was not increased, this observation suggests rapid DNA restoration by repairing enzyme activities due to the temperature-risen reaction rate in the Panc1 radio-resistant cell line (Figure 19(F)). Noteworthy, mEHT also destroys the tumor stem cells in pancreatic adenoma-carcinoma cell lines (Panc1 and Capan) [97], as well as dramatically reduced cancer stem cells (CD133+) population in glioma cell lines (U87-MG and A172) after mEHT treatment [86].



D.

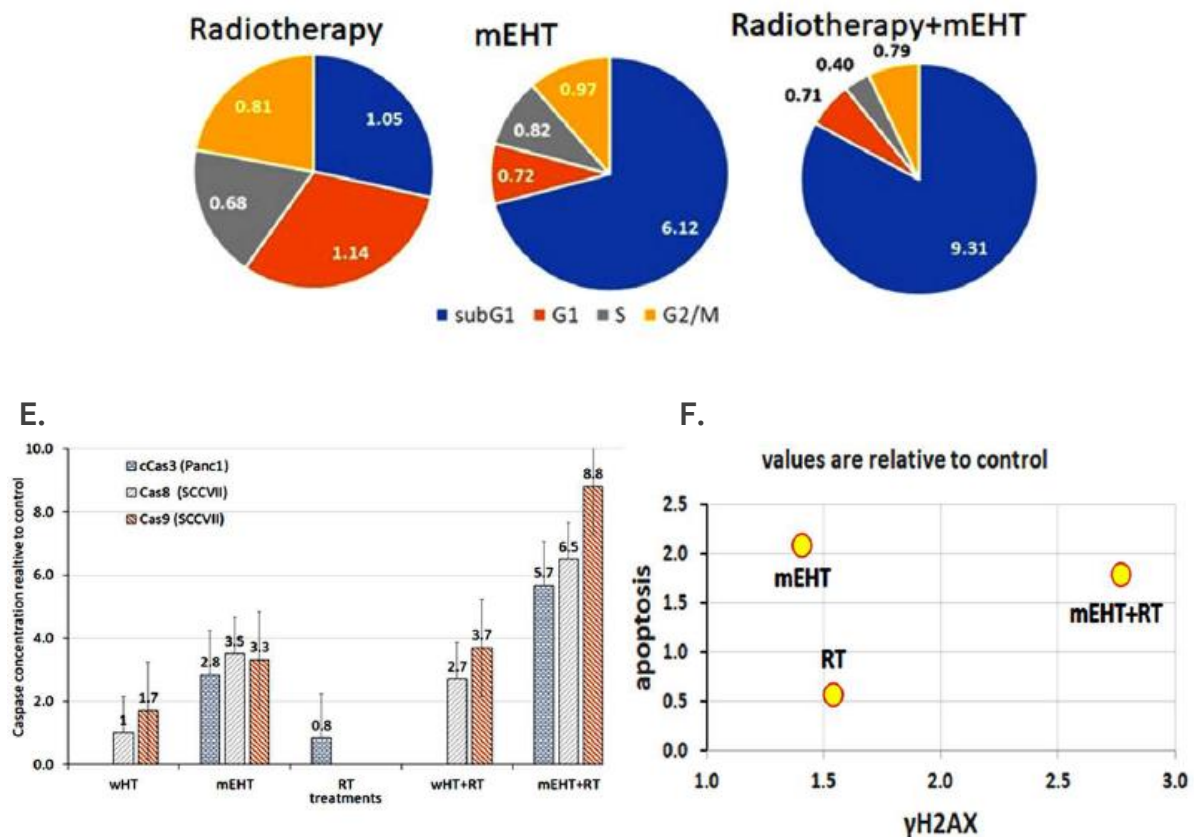


Figure 19. Various experiments of RT + mEHT combined therapy. (A.) Apoptosis in different temperatures with various treatments [92]. (B.) Autophagy in different temperatures with the various treatments [92]. (C.) Apoptosis and autophagy ratio. (D.) Effect on cell cycle of the therapies [81]. (E.) Caspase concentration relative to control Panc1 cell line [81], SCCVII cell line [92]. (F.) apoptosis dependence on γ H2AX expressions [81].

Complementary applications with chemotherapy also have advantages observed in vitro. The mEHT highly promotes the intake of liposomal doxorubicin (Lipodox), together with an effective decrease of cellular viability in mEHT + Lipodox combined therapy in various cell lines (Figure 20(A) [89]. The doxorubicin damages DNA and interferes with DNA replication. MEHT improves the release of doxorubicin compared to the same temperature wHT treatment Figure 20(B). Effective cell cycle arrest was observed in the C26 cell line in a combined mEHT + Lipodox combination [83] Figure 20(C), but mEHT dominates the apoptosis indicated by subG1 quantity.

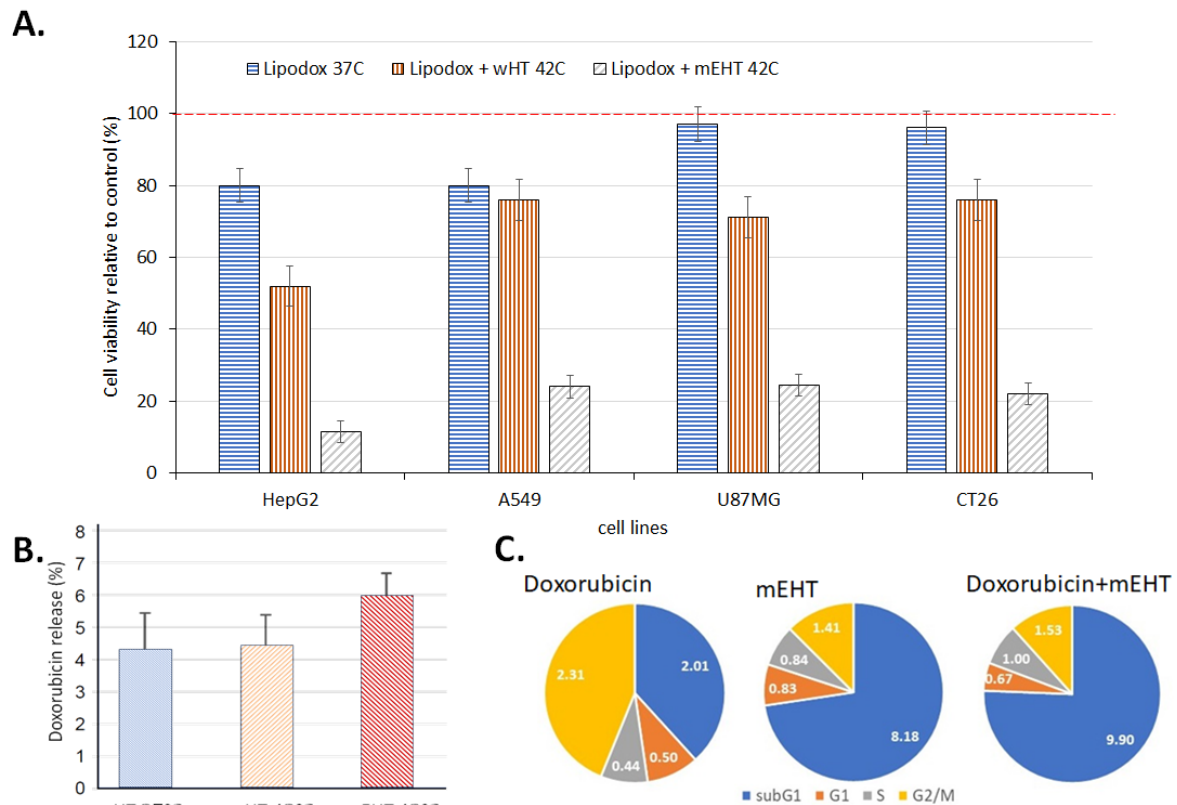


Figure 20. Complementary application of mEHT with liposomal doxorubicin (Lipodox). (A.) Cell viability for different cell lines [89]. The addition of mEHT significantly improved all cases. (B.) The doxorubicin release of Lipodox in a medium heated by wHT and mEHT [89]. (C.) The cell cycle changes by mEHT application [83].

Electrical stimuli increase the penetration of drugs like doxorubicin, and the nonthermal processes could bypass the multidrug resistance [103] *in vitro*. Further proof supports the theoretically established molecular models concentrating on the synergic thermal and nonthermal membrane effects. One obvious process of the thermal and nonthermal processes is loading the system with various stresses, inducing systemic and cellular reactions.

The addition of the nonthermal effect to the conventional thermal one significantly modifies the gene expression of the U937 cell line in comparison to wHT and mEHT samples [87] Figure 21. The strong upregulations are in the stress protein genes and their connected processes.

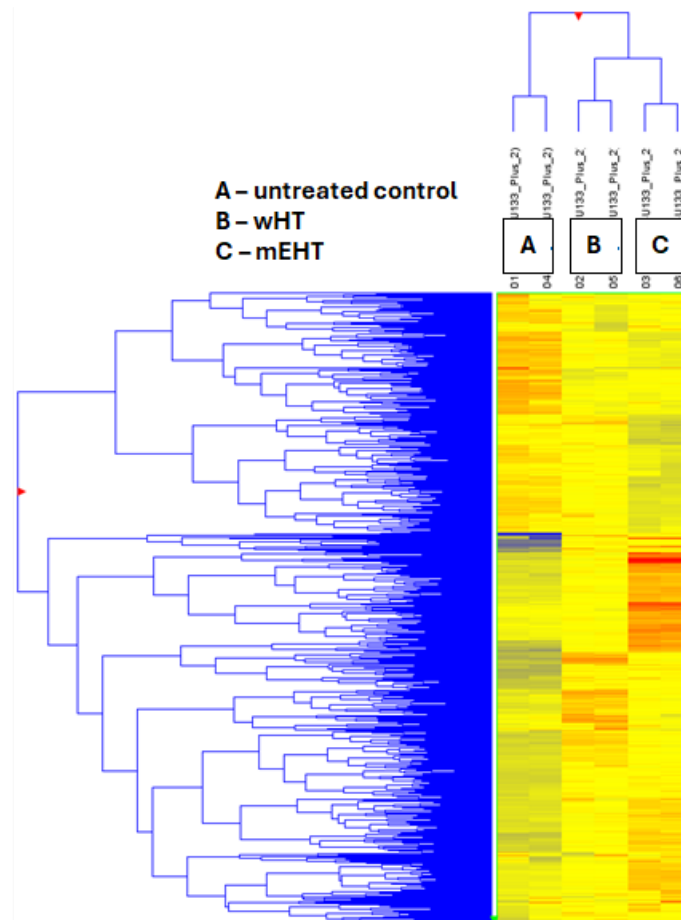


Figure 21. Gene comparison of the control, wHT, and mEHT-treated cell lines. The significant differences show the impact of the nonthermal effects.

The system develops stress- (or heat-shock) proteins (HSPs). HSPs are molecular chaperones playing a role in protein maturation or degradation. HSPs can refold and repair the stress-damaged unfolded proteins or inhibit their denaturation in response to stress. The function of HSPs is not limited to the protection on a cellular basis. They have a role in protecting multicellular structures and may even participate in systemic processes in the organism. Their intracellular chaperoning maintains the balance between the intracellular proteins. HSPs regulate apoptosis and protect the cells from mechanical, chemical, thermal, or electromagnetic stressors. However, it has multifaceted behavior as most actors in the complex living system could promote or inhibit inflammation, tumoral growth, and immune processes. The gene chip heat map and ingenuity pathway analysis (IPA) on the U937 human lymphoma cell line (Figure 22 [87]) revealed some activated stress responses differentiating wHT and mEHT processes. Notably, the cytoprotective functions of the thermal impact (wHT) show strong activation of HSP70, HSP90, and BAG3 genes. The cytoprotective action of HSPs and proliferation promoter BAG3 could play a role in the resistance to heat therapy and support the further development of tumors. The activated HSPs arrest apoptosis by interfering with caspase activation, directly inhibiting apoptosis and indirectly blocking tumor distortion. The effect of mEHT is different. There is no such massive upregulation of HSPs as in wHT, and the BAG3 is downregulated, allowing the apoptotic processes. According to the HSP protein measurements, the HSPs linearly develop after the heat treatment for days, demonstrating the intensive continuous processes after the treatment. The slope of HSP development grows approximately twofold higher with mEHT than with wHT at the same

temperature, indicating the difference in the posttreatment processes caused by the pure thermal and synergic thermal nonthermal impact.

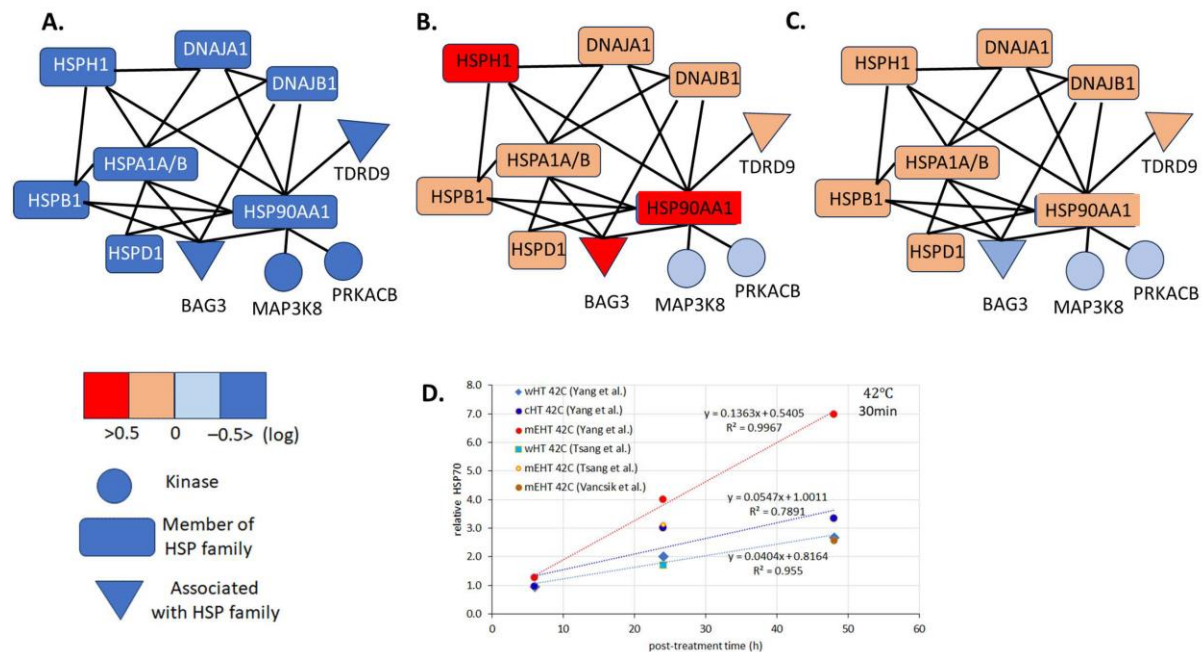


Figure 22. The IPA analysis of gene chip heat map [87]. A specific cytoprotective gene network (heat shock proteins, HSPs) was activated in the case of wHT treatment but not in mEHT-treated samples. (A.) Unheated control, (B.) wHT result. (C.) mEHT result. (D.) Increase of HSPs by posttreatment time [77] [83].

The metabolic profiles of the targeted malignant cells have elevated glycolysis [104]. The efficacy of mEHT may correlate with the tumor metabolic profile by the targeted selection [105]. Different molecules majorly involved in the mEHT processes were measured as having a key influence on the cancer cells' fate by mEHT Figure 23. The reactive oxygen species (ROS) are more than doubled compared to the same temperature wHT and cHT [77] Figure 23(A). The calreticulin (CRT) is also significantly increased by mEHT Figure 23(B). Despite the simple thermal effect (wHT and cHT treatments) not showing such improvement. Noteworthy is that the plane wave capacitive coupling (cHT) method works like wHT in ROS level and CRT, which shows its thermal dominance in homogenous thermal heating, while on the contrary, selecting nonthermal processes massively increases the ROS and CRT products. This proves the nonthermal effects of mEHT on the U937 cell line well. The mRNA changes for inflammatory chemokines CXCL9,10,11 and matrix metalloproteinase (MMP-2) in A2058 melanoma cell lines [108] Figure 23(C), Figure 23(D). also supports the advantages of mEHT. The chemokines bind to a specific receptor called CXCR3 on immune cells, particularly T cells, attracting them to the tumor site and potentially promoting an immune response against the cancer. However, suppressing CXCL9, 10 while increasing CXCL11 could support tumor progress. The increase in MMP-2 signifies a growth in the activity of an enzyme that breaks down components of the extracellular matrix (ECM), a double-edged sword that promotes or blocks promote or block cancer growth. The mEHT lowers the mitochondrial membrane potential in approximately a quarter of malignant cells. At the same time, the wHT (only thermal process, changes only a tiny fraction of cells Figure 23(E), which could be connected to

the increased ROS level and promotes the cytochrome c (Cyt-c) secretion, a point of nonreturn of apoptosis, may compensate well for the opposite effect of inflammatory cytokines and MMP-2. The decreased membrane potential of mitochondria [87] well supports the mitochondria-associated apoptotic process. The mEHT induces the Ca^{2+} influx with the assistance of E2F1 [86], which regulates the HSPs without heat shock [106], supporting the possible factors of the nonthermal effect of applied electric current. The five-fold increase in the Ca^{2+} positive cells relative to the same temperature wHT Figure 23(F). strongly supports the high impact of nonthermal processes. The Ca^{2+} acts as a second messenger inside cells. Depending on its concentration and location within the cell, it triggers various cellular responses. Its massive and sustained influx can contribute to cell death and promote apoptosis in the mitochondria-dependent intrinsic signal pathways. The wHT represented thermal impact also forces the increase of the Ca^{2+} ion concentration in the cell, but mainly from the internal sources, processes in the cytosol ER and other cellular organelles, and some influx by the heat-sensitive transient receptor potential vanilloid receptor (TRPV), which also senses the same temperature for action.

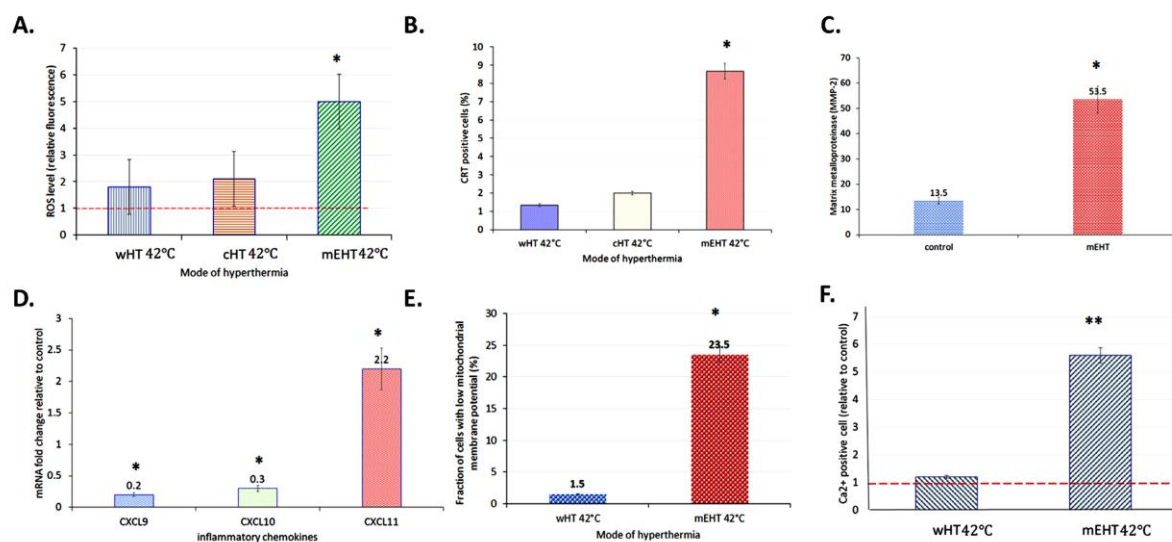


Figure 23. Involvement of various molecules is measured 24 h posttreatment of mEHT. (A.) ROS level, HepG2 cell line [77]. (B.) Development of inflammatory chemokines A2058 cell line [108]. (C.) MMP-2 development A2058 cell line [108]. (D.) The calreticulin expression HepG2 cell line [77]. (E.) The fraction of cells with lowered mitochondrial membrane potential U937 cell line [87]. (F.)

The mEHT significantly triggers the Ca^{2+} influx, while only thermal processes make minor changes, U937 cell line [87].

The only thermal effects (wHT) induce only a minor influx, but the synergy of thermal and nonthermal processes massively causes a significant Ca^{2+} influx to malignant cells. The temperature affected the internal. Ca^{2+} release can not be the origin of such considerable ion concentration because the temperature did not change. Noteworthy nonthermal effects cause the reconstruction of the intercellular E-cadherin/ β -catenin connection, allowing the regular networking of the cells [77] [75] [107] which may be a factor in the inhibition of the proliferative activity of the tumor.

Detailed molecular analysis of the U937 cell line with western blot (WB) [87], [95] shows the FAS starting external apoptotic path through cCas-8, which signal had pointed expression

enhancement from 1 h to 3 h after mEHT treatment. In contrast, the wHT-treated cell line did not show a similar effect. The FAS, p-JNK N-terminal kinases (JNK), and p-ERK signaling pathways dominate the apoptotic pathway, also developing markedly for 1 h to 3 h posttreatment in mEHT but not in wHT, well demonstrating the synergic nonthermal component to the pure thermal delivery by wHT. The ingenuity pathway study shows these remarkable differences in Figure 24. The significant difference between the only thermal wHT and a nonthermal addition with mEHT also determines these results. Contrary to the temperatures in both heating methods being the same, the observed difference in pathway activations is likely primarily due to the electric field effects [87]. The IPA demonstrated a specific gene network containing cell death-related genes, such as EGR1, JUN, and CDKN1A.

The expression levels of these genes were elevated only in the cells treated with mEHT. However, complex networking must explain how various double-faced molecular actions can become proapoptotic activity. The CDKN1A gene (coding p21 protein), a critical cell cycle regulator, plays a critical role in cell cycle control and DNA repair. It is a crucial tumor suppressor gene and has a central role in that part of IPA (Figure 24(C)). Its high upregulation is observed only in the mEHT process despite the same thermal effect in wHT treatment.

The further remarkably upregulated genes, the EGR1, JUN, TNF, and SMAD, all have multifaceted behavior. The importance of the extracellular processes is that the ERK gene (extracellular signal-regulated gene) is highly expressed and activates CDKN1A, TNF, JUN, EGR1 IER3, and MAFF (Figure 24(F)), which are actively upregulated in death-related genes (Figure 24(C)). With their multifaceted behavior, the main processes that could actively help the tumor death are various. TNF can directly kill cancer cells but also may promote tumor metastasis. TNF superfamily contains the TRIAL receptors, which are excited by nonthermal mEHT impact [17], [14].

Despite its complex role, TNF may be an immunostimulant for certain cancers. The ERK (Extracellular signal-regulated Kinase) is another player with a double-edged sword role in cancer. When the ERK pathway is abnormally activated, it can lead to uncontrolled cell growth. Still, the very high levels of ERK activity can also trigger cell death or senescence, potentially acting as a tumor suppressor. In some cancers, EGR-1 acts as a tumor suppressor, but it may have the opposite effect, promoting tumor growth. JUN protein also plays a complex role in cancer, with the potential to both promote and suppress tumors depending on the context. Its overexpression can contribute to uncontrolled cell growth but can also have antitumor activity; it might trigger cell death (apoptosis).

The IPA-involved SKIL, IER3, MAFF, SMAD, and HBEGF molecules are also a double-edged sword. Still, commonly, they may promote extracellular rearrangements, which may help the metastases or cell apoptosis. The mEHT turns these complex processes to the positive side, assisting the apoptosis and tumor degradation.

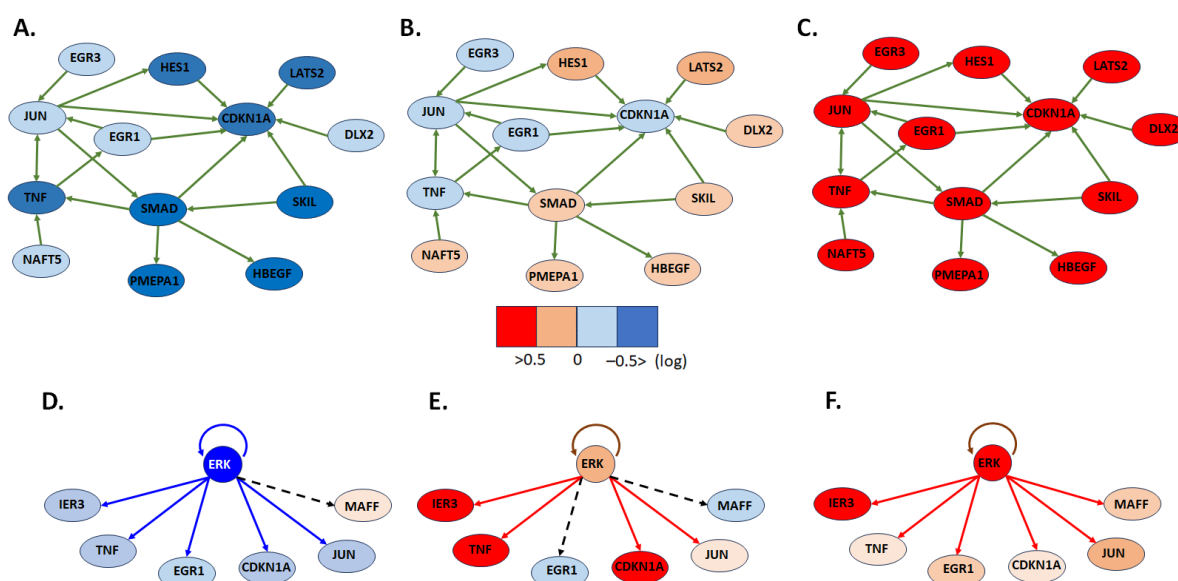


Figure 24. The gene maps and IPA analysis show significant differences in the gene regulation between wHT and mEHT-treated U937 cell lines at the same 42°C [87]. (The edge (form) labels of molecules are not shown.) (A.) and (D.) controls are 37°C (B.) and (E.) the wHT and (C.) and (F.) the mEHT results. Both maps show the upregulation of the CDKN1A and ERK pathways from wHT to mEHT.

Another investigation [86] reveals the number of mRNA transcripts up- or down-regulated in U87-MG or A172 cells after mEHT treatment. The majority of the extra up and downregulated genes differ by U87-MG and A172 cell lines, but 19 and 15 up and down-regulation overlapped, respectively [86].

6. CONCLUSION

The thermal and nonthermal differences clearly appear in the comparative studies of only thermal water bath hyperthermia (wHT), and electrothermal (mEHT) experiments, shown in Figures 12–24. The advantage of the combined thermal and nonthermal treatment is proven. The thermal conditional basis works similarly to ionizing radiation combination, when combined with the nonthermal electric field. The in vitro experiments (Figure 25) verified the in silico calculations and the proofs of the concept using phantoms of modulated electro-hyperthermia. The robust apoptosis of the malignant cells is shown on very different cell lines, such as mEHT, which is significantly far ahead of the pure thermal effects of water bath treatment. These observations significantly impact nonthermal processes and may open new ways to evaluate the effect of hyperthermia with nonionizing radiations. In this frame, ionizing radiation (radiotherapy, RT) and nonionizing hyperthermic oncology could be united, considering the molecular effects of RT on DNA strands, in principle is the same nano-selection as the mEHT impacts on the nanoscopic localization transmembrane proteins. While the RT is focused on the break of chemical bonds, and the thermal effects have only a tiny portion of the energy absorption, in nonionizing hyperthermia, the thermal effects boost the nonthermal molecular actions.

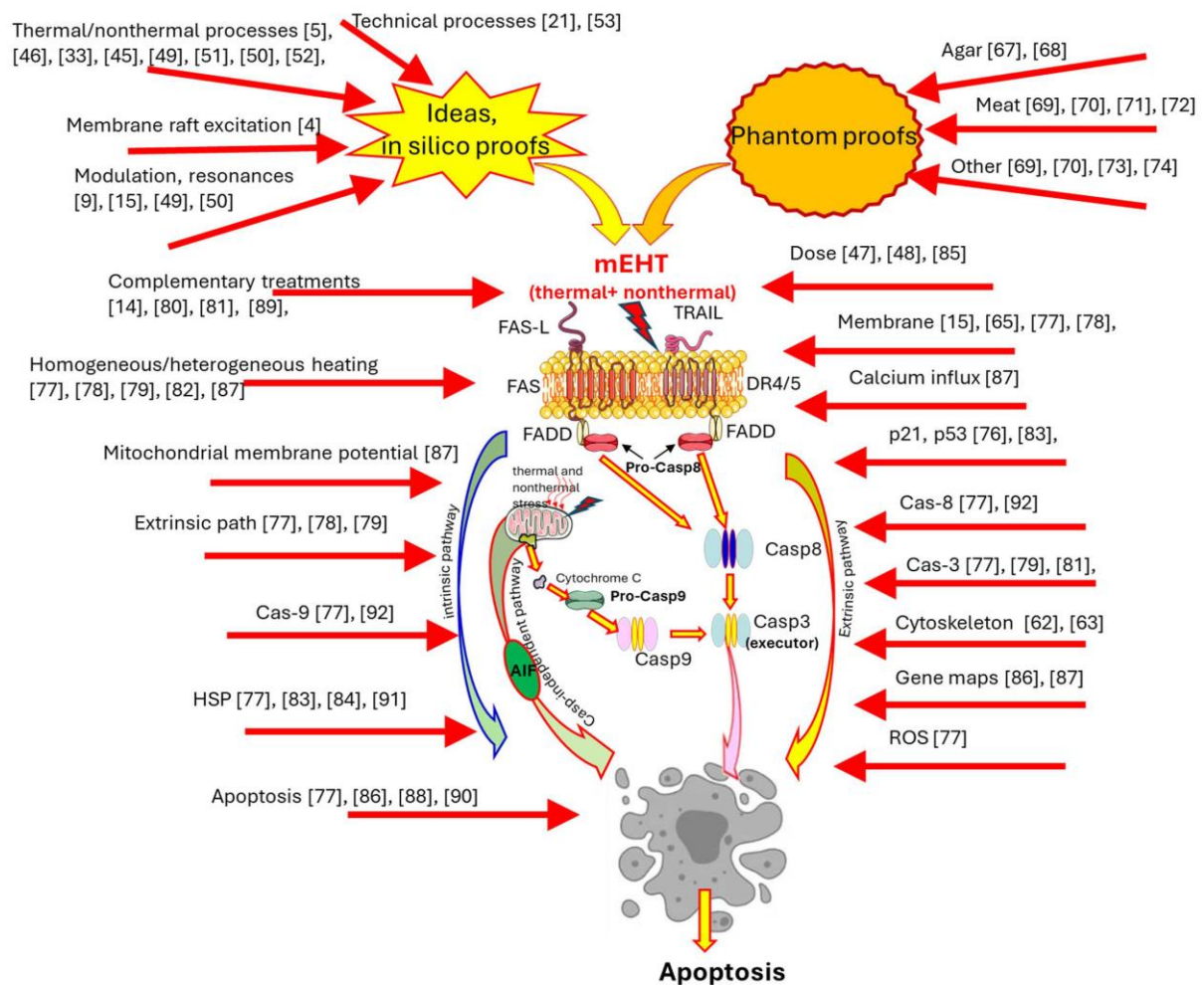


Figure 25. The structure of publications is discussed in this paper.

The following parts of this series will show the in vivo preclinical results, which strongly verify the above conclusion, and the clinical studies finally validate the mEHT concept.

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Conflicts of Interest

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

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