OPINION

Thermal ablation of tumours: biological mechanisms and advances in therapy

Katrina F. Chu and Damian E. Dupuy

Abstract | Minimally invasive thermal ablation of tumours has become common since the advent of modern imaging. From the ablation of small, unresectable tumours to experimental therapies, percutaneous radiofrequency ablation, microwave ablation, cryoablation and irreversible electroporation have an increasing role in the treatment of solid neoplasms. This Opinion article examines the mechanisms of tumour cell death that are induced by the most common thermoablative techniques and discusses the rapidly developing areas of research in the field, including combinatorial ablation and immunotherapy, synergy with conventional chemotherapy and radiation, and the development of a new ablation modality in irreversible electroporation.

Thermal or energy-based ablation of tumours is the local application of extreme temperatures, which can be either high or low, to induce irreversible cell injury and ultimately tumour apoptosis and coagulative necrosis. Percutaneous energy-based ablation has been used for the treatment of many tumour types, including liver, kidney, lung and bone cancers, as well as soft-tissue tumours of the breast, adrenal glands, and head and neck. This technology rapidly advanced in the 1990s, after the advent of cross-sectional imaging made percutaneous, image-guided procedures not only possible but also commonplace[1,2] (FIG. 1 (TIMELINE)). Now, percutaneous thermal ablation is primarily used for the treatment of small, unresectable tumours or for patients who are poor surgical candidates.

Thermoablative technology offers several advantages over surgical resection: most notably, lower morbidity, increased preservation of surrounding tissues, reduced cost and shorter hospitalization times[3], as well as intra-procedural monitoring by visualization, not to mention the ability to treat patients who are not candidates for conventional therapies. However, common disadvantages include incomplete ablation[2,4], disease recurrence and inferior outcomes — although efficacy, functional outcomes and improvements in mortality over conventional treatment methods vary substantially from modality to modality and among different tumour types. No large randomized controlled trials have yet been undertaken to directly compare outcomes of thermal ablation versus surgical resection or radiation[5]. Regardless, given that tumours are increasingly being detected at an earlier stage[6]; given that the proportion of elderly patients is increasing; and given that the clinical use of minimally invasive, image-guided thermal ablation is increasing overall, a better understanding of the biological factors that might modify treatment response is crucial.

Currently, the most commonly used thermal techniques, and the main focus of this article, are radiofrequency ablation (RFA) and microwave ablation (MWA), which are high-temperature-based modalities, and cryoablation, which is a low-temperature-based modality. Newer technologies, such as high-intensity focused ultrasound (HIFU) and laser ablation are conceptually similar to high-temperature-based ablation but are less well studied. HIFU is the only non-invasive hyperthermic modality. It uses multiple ultrasound beams and focuses them on a selected focal area to generate temperatures of up to 60 °C using acoustic energy, which causes coagulative necrosis[5]. HIFU also causes acoustic cavitation, which occurs when acoustic pressure causes expansion and contraction of gaseous nuclei in cells, thereby leading to the collapse of the cell and nuclear membranes, the mitochondria and the endoplasmic reticulum[5]. Laser ablation generates electromagnetic heating, as do RFA and MWA, with the advantage of laser precision and efficiency during laser ablation. However, because light is easily scattered and absorbed, this modality has a limited tissue penetration and hence ablates very small areas of about 1–2 cm^2 (REF. 2). In addition, irreversible electroporation (IRE) is one of the newest technologies for tumour ablation. Although it does not use thermal energy as its primary mechanism, we highlight it because of the recent exciting research on the topic. IRE generates an electric field by using multiple pulses of an intense electrical current to cause irreversible cell membrane damage and cell death[7].

A rapidly growing area of research in energy-based ablation techniques is based on the idea of immunomodulation that is activated by these therapies, which could contribute yet another mechanism of tumour cell death and destruction. Recent reports[8–11] that describe an unexplained, spontaneous regression of untreated distant metastases after thermal ablation of the primary tumour have generated interest in a possible systemic antitumour immune response induced by focal thermal ablation. Spontaneous regression outside of the treatment field after the application of local therapy has been observed using other modalities, such as radiotherapy[12]. This has roused a whole new area of cancer research and may have implications for immune enhancement or combinatorial treatments.

In this Opinion article, we discuss the literature on the mechanisms of tumour cell death that are induced by the most common thermoablative techniques. We focus on the burgeoning literature on ablation-related immunomodulation, examine the role of image-guided thermal ablation in combinatorial therapies and discuss one of the newest modalities in image-guided tumour ablation.

Mechanisms of energy-based cell death

Hyperthermic injury. RFA and MWA, as well as laser ablation and HIFU, cause focal hyperthermic injury to ablated cells, which affects the tumour microenvironment and damages cells at the membrane and subcellular levels. The process of tumour destruction occurs in at least two phases, through direct and indirect mechanisms[13] (FIG. 2).

Heat-ablated lesions can be thought of as having three zones[2]: the central zone, which is immediately beyond the application tip and which undergoes ablation-induced coagulative necrosis; a peripheral or transitional zone of sublethal hyperthermia, which mostly occurs from thermal conduction of the central area that is either undergoing apoptosis or recovering from reversible injury; and the surrounding tissue that is unaffected by ablation.

Direct cellular damage occurs at several levels, from the subcellular level to the tissue level, and it depends on the thermal energy that is applied, the rate of application and the thermal sensitivity of the target tissue[13]. Our current understanding of the secondary effects of hyperthermia is often extrapolated from literature on low-temperature hyperthermia, wherein cell lines or tissues are exposed to uniformly low temperatures for long periods. At temperatures of around 40–45 °C, irreversible cell damage occurs only after prolonged exposure (from 30 to 60 minutes). At temperatures of above 60 °C, the time that is required to achieve irreversible damage decreases exponentially. Inactivation of vital enzymes is an initial feature of injury. Above 60 °C, rapid protein denaturation occurs, which is immediately cytotoxic and leads to coagulative necrosis[13].

Changes to cell membrane integrity were first considered to be the main cause of hyperthermia-induced cell death. Rising temperatures have been shown to change cell membrane fluidity and permeability, and this causes dysfunction of actin filaments and microtubules, thereby leading to an impairment of facilitated diffusion across the cell membrane[14]. Metabolite accumulation and intracellular fluid shifts subsequently cause cytolysis. However, these changes in membrane stabilization might be the end result of subcellular processes, and they do not have a direct correlation with rising temperature[13].

However, mitochondrial dysfunction has been well correlated with heat-induced injury. High temperatures might promote the leakage of protons through the inner mitochondrial membrane[15]. Major ultrastructural changes can be seen minutes after heat injury; these include vesicularization of the mitochondrial cristi, mitochondrial swelling and the formation of dense bodies[16].

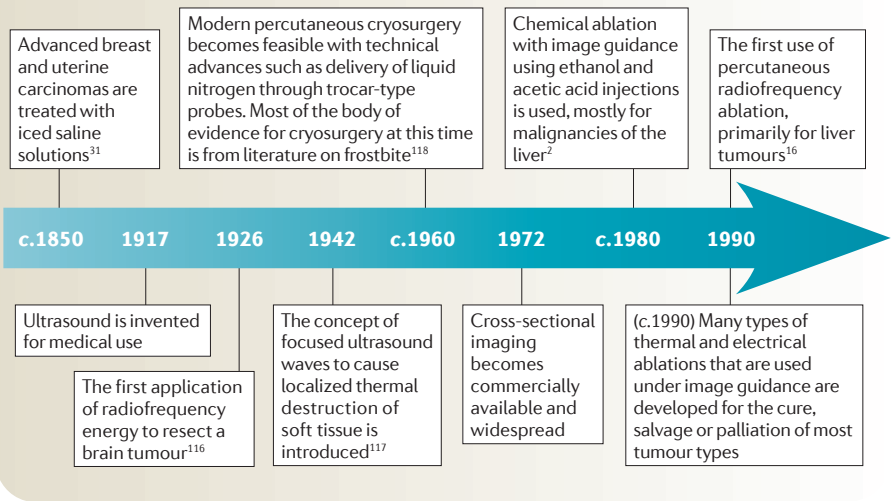
Moreover, DNA replication is rapidly inhibited by hyperthermia, which suggests a heat-mediated reproductive cell death. This could occur by denaturation of the crucial replication enzymes, such as DNA polymerase α, which is responsible for semiconservative DNA replication, and DNA polymerase β, which is responsible for DNA repair synthesis[17]. Alternatively, because DNA replication remains suppressed after heat cessation and the recovery of protein synthesis, another possible mechanism is denaturation of the polymerase substrate chromatin. Heat-induced abnormal condensation of non-histone nuclear matrix proteins has been posited to physically obstruct DNA replication and repair enzymes[17]. Other proposed intracellular mechanisms of heat-induced injury include the disruption of RNA synthesis, the release of lysosomal enzymes and the impairment of the Golgi apparatus[13,14]. It is also important to note that tumour tissue has been shown to be more thermosensitive than normal tissue. This may be related to increased tumour metabolic stresses above normal levels, the reduced heat-dissipating ability of the tumour and its acidic interstitial environment[13].

Indirect or delayed cellular damage also occurs after thermal ablation. Even after cessation of thermal ablation, delayed heat-induced injury is apparent. This has been shown in both preclinical and clinical studies and has several proposed mechanisms, including induction of apoptosis, vascular damage that causes ischaemia, ischaemia–reperfusion injury, lysosomal contents that are released during tumour necrosis or from invading granulocytes, cytokine release and further stimulation of an immune response[13,14].

Radiofrequency ablation. Percutaneous RFA is the direct placement of one or more radiofrequency electrodes into the tumour tissue by using ultrasound, computed tomography (CT) or magnetic resonance guidance; the initial success of RFA in the treatment of hepatic malignancies has expanded its clinical or experimental use to neoplasms of the kidneys, breast, bone and lungs[18,19]. Temperatures between 60 °C and 100 °C are generated by a high-frequency alternating current, which induces frictional heating when the ions in the tissue attempt to follow the changing directions of the alternating current[11]. This frictional heating (also known as 'resistive' heating) causes cell injury by the above-stated hyperthermic mechanisms and subsequent coagulative necrosis. Interestingly, temperatures >100 °C are less effective, as the desiccation that results at these temperatures, which manifests as water vapour and burnt tissue, increases the tissue impedance and therefore limits further electrical conduction through the remaining tissue[20]. Additionally, cytotoxic temperatures are difficult to maintain if the ablated tumour is near large blood vessels. This heat-sink effect is a commonly described limitation of RFA and occurs when heat that is absorbed by flowing blood or air is carried away from the area of ablation, thereby dissipating the hyperthermia and decreasing RFA efficacy; because of this, tumour tissue that is adjacent to vasculature is less susceptible to thermal damage[21,22].

In the transitional zone, which occurs adjacent to the central area of coagulative necrosis, studies have reported inflammatory infiltrates that include neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, as well as B cells and T cells that are specific to the ablated tissue[23–25]. These immune cell subsets have also been observed in distant, untreated tumours[26], as well as peripherally in the bloodstream[27–29] in both patients and animals; these results suggest an overall immune activation by RFA. Mechanical cell damage that is caused by heat-induced necrosis releases various immunogenic intracellular substrates — RNA, DNA, heat shock proteins (HSPs), uric acid and high mobility group protein B1 (HMGB1)[30,31] — all of which activate innate immunity and can lead to acquired responses.

Timeline | **Development and evolution of image-guided thermal ablation**



Pro-inflammatory cytokines that are released from the ablated tissue or tumour cells, as well as from the disruption of local extracellular matrix and tissue components such as fibrinogen, hyaluronic acid and endothelial cells trigger the release of additional cytokines, chemokines and vascular adhesion molecules[31]. Levels of serum interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis factor-α (TNFα) have all been shown to increase after RFA (on the timescale of hours to days)[28,29,32,33].

Increasing evidence supports that induction of HSP70 by RFA has a key role in stimulating the antitumour immune response[34–38]. HSPs have diverse functions and are abundantly expressed by tumour cells, and they are secreted into the extracellular space by tumour cells, by virally infected cells or during cell necrosis[39]. Intracellular HSPs protect against tissue injury by inhibiting apoptosis[40], whereas extracellular HSPs are involved in various immunological processes, as an antigen chaperone to antigen-presenting cells (APCs) and as a danger signal to the immune system by activating DCs[23,41–43]. In animal models, preparations of HSPs that were derived from tumour cells or virally infected cells induced antigen-specific immunity[44,45]. In addition to animal studies that have shown increased expression of HSP70 in tumour cells[38], the levels of HSP70 in human liver biopsy material[35] and in the sera of cancer patients[46] have been shown to be significantly higher after RFA. Furthermore, increased serum levels of HSP70 correlated with better survival in patients who were treated with RFA[46]. How RFA increases the expression of HSP70 might be related to the necrosis that is induced, although only weak correlations between the levels of applied RFA energy and tumour necrosis have been shown[47] and no clear mechanism has been elucidated.

A decrease in $CD4^+$ $CD25^+$ forkhead box protein P3 (FOXP3)$^+$ regulatory T cells (T_{Reg} cells) in response to RFA has also been noted[29], and this decrease indicates that one of the mechanisms of tumour recognition may be a reduced peripheral tolerance to tumour antigens. Acquired immunity is activated in the form of enhanced antitumour humoral and cell-mediated responses, as shown in various studies in animal models and cancer patients. Increased levels of tumour-specific T cells have been detected in post-RFA cancer patients[24,48] and confers increased tumour-free survival in certain patients[48]. These T cells can also cause resistance to tumour rechallenge[23] in animal models.

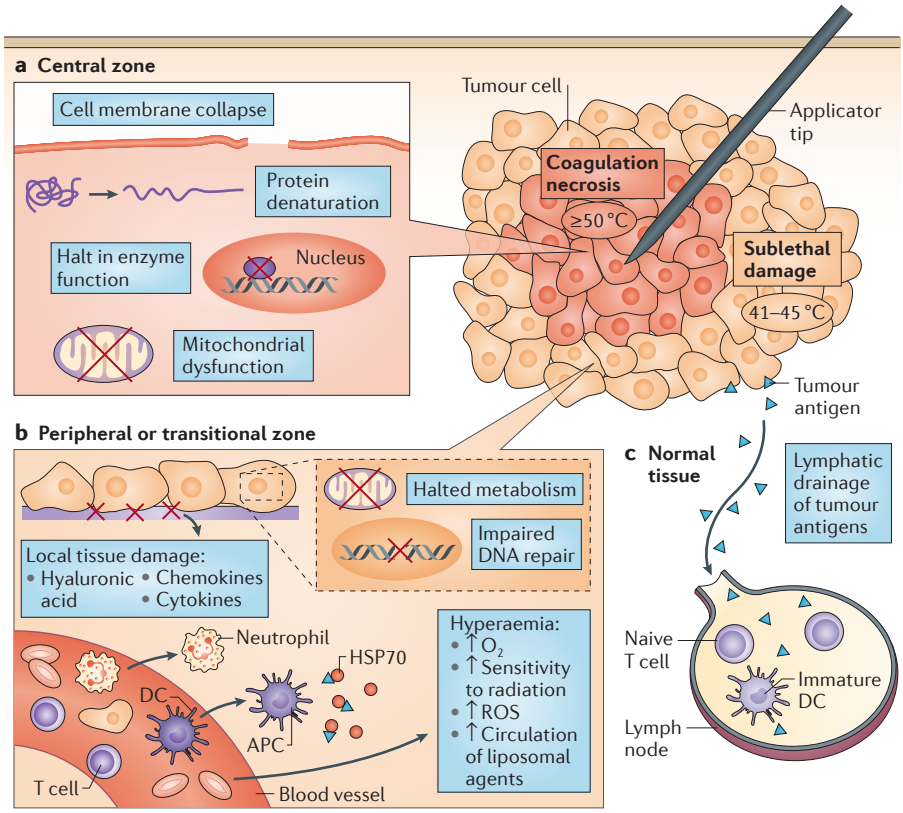


Figure 2 | **The zones of hyperthermic ablation.** The applicator tip is surrounded by three zones. **a** | The central zone undergoes coagulative necrosis at temperatures ≥50 °C. Cell membrane collapse, protein denaturation, a halt in enzyme activity and DNA polymerase function, and mitochondrial dysfunction all occur[13]. **b** | The peripheral or transitional zone has a steep negative temperature-gradient. At temperatures between 41 °C and 45 °C there is still heat-induced injury, but it is sublethal and reversible. Metabolic functions might be deranged or halted, and cells in this zone are vulnerable to further injury; for example, radiation-induced inhibition of DNA repair and cell recovery can eliminate already susceptible cells. The peripheral zone has increased blood flow (hyperaemia), and this results in increased oxygenation that sensitizes the tumour tissue to radiation and may increase the formation of reactive oxygen species (ROS; e.g., free radicals). Increased blood flow in this area facilitates the accumulation of liposomally-delivered chemotherapeutic agents. Damaged local tissue exposes hyaluronic acid and markers of endothelial injury, which stimulates the expression of vascular adhesion molecules and chemokines that attract immune cells. This zone contains the most inflammatory infiltrates, including neutrophils, macrophages, natural killer cells, dendritic cells (DCs), as well as $CD4^+$ and $CD8^+$ T lymphocytes. Intracellular necrotic debris stimulates phagocytosis, and tumour cells are engulfed by antigen-presenting cells (APCs). Heat shock protein 70 (HSP70) can chaperone antigens to APCs. **c** | In the normal surrounding tissues, blood vessels cause a heat-sink effect, which dissipates the elevated temperature and decreases the ablation efficacy. Tumour antigens that are released after necrosis drain to nearby lymph nodes, where they can stimulate immature DCs and naive T cells[43].

Microwave ablation. Like RFA, MWA uses electromagnetic waves to generate heat and also kills cells by the aforementioned mechanisms of direct hyperthermic injury. An electromagnetic field, which is typically between 900–2500 MHz, is created through an intratumourally placed antenna. This field forces the polar molecules with intrinsic dipoles — predominantly water — within the tissue to continuously realign with the oscillating electric field[49]. This phenomenon is known as dielectric hysteresis, or rotating dipoles. The rotation of the molecules increases their kinetic energy, thereby elevating the temperature of the tissue. In contrast to RFA, MWA does not rely on electric currents and conduction through tissue, so temperatures >100 °C are usually administered without the concern that desiccation will disrupt therapeutic delivery. MWA is therefore more suitable for tissues with higher impedance, including lung and bone, and for tissues with a high water content, such as solid organs and tumours[49].

MWA has several advantages over RFA, including the ability to achieve better heating of larger tumour volumes and a lower susceptibility to heat-sink effects because

microwave systems are faster and more efficient[50]. During RFA, the zone of active heating is limited to a few millimetres around the active electrode, and the remainder of the treated tissue is heated by thermal conduction. By contrast, MWA at certain frequencies can heat tissue up to 2 cm away from the antenna[2]. Another advantage of MWA is the ability to use multiple antennas to amplify the ablative effect, which enables larger or multifocal tumours to be ablated simultaneously. Phasing the electromagnetic waves constructively, the heat generated is proportional to the square of the number of antennas[49]; therefore, simultaneous activation of multiple antennas results in a synergistic (rather than additive) increase in lesion size[50]. This synergistic capability is not available with RFA, as multipolar radiofrequency fields would need to be continuously switched between pairs of monopolar electrodes. However, MWA systems are more cumbersome than RFA and use larger cables. In addition, the antenna is prone to overheating, which necessitates a cooling mechanism to protect the superficial structures along the antenna[49].

In terms of delayed or indirect mechanisms of tumour destruction, MWA is a weak stimulator of local inflammation, as well as innate and acquired antitumour immunity. The induction of pro-inflammatory cytokines, including IL-1 and IL-6 (REF. 33), by MWA is minimal compared with that by the other ablative techniques, as is the expression of HSP70 (REF. 51). Even so, the extent of immune cell infiltrates in the ablated tissue is inversely correlated with clinical outcome — specifically, overall survival and risk of local recurrence[52] — in a statistically significant manner.

Cryoablation. In contrast to the hyperthermic techniques, cryoablation uses cold injury to kill tumours. This technique has a longer history than the other energy-based ablative methods and was first used to treat breast and uterine cancers in the 1840s, before it gained traction as a modern technique in the 1960s, when systems that were capable of delivering liquid nitrogen through trocar-type probes were developed[31] (FIG. 1 (TIMELINE)). Since then, it has been used for cancers of the retina, skin, prostate, kidney, liver, breast, lung and bone[31].

Cryoablation uses liquefied gases that cool as they expand, such as argon. Gas expansion in a small chamber at the distal end of the cryoprobe creates a heat sink and reduces the temperature to as low as –160 °C when argon evaporates[2]. The temperature that is necessary for cell lethality is between –20 °C and –40 °C, and studies have shown that this temperature needs to persist to 1 cm beyond the tumour periphery to ensure complete ablation[53,54]. Such a size requirement limits the tumours that can be targeted. Of note, various biological mechanisms have been described for cryoablative injury and occur in different zones of the cryolesion. They fall under four main categories: direct cell injury, vascular injury and ischaemia, apoptosis, and immunomodulation[55] (FIG. 3).

> “Understanding the biological factors that help or hinder thermal ablation has tremendous importance for enhancing or augmenting outcome.”

Direct cellular injury occurs when freezing causes cellular dehydration. The water in the extracellular compartment freezes before the water in the intracellular compartment, which is protected by the lipid bilayer. This leads to a higher extracellular solute concentration, which causes an osmotic gradient, fluid efflux, cell shrinkage and distortion of the plasma membrane[56]. The cell dehydration and high extracellular solute concentration is called the solution-effect injury. It is enhanced by ice crystal formation within the cells, which further injures the integrity of organelles and the cell membrane. During the thaw, the intracellular compartment becomes hypertonic, and fluid shift causes the cell to burst[31].

Vascular injury occurs when cryoablation causes endothelial damage to the microvasculature, which leads to platelet aggregation, vascular stasis and microthrombosis. Vasoconstriction occurs in response to cooling temperatures, thereby also causing vascular stasis. This results in ischaemic death to the targeted area, and this furthers the coagulative necrosis.

Direct cold-induced coagulative necrosis occurs at the centre of cryoablative lesions, whereas apoptosis has been observed at their periphery[57]. The sublethal temperatures in the peripheral zone can cause some cells to activate apoptosis, as shown in animal studies[58,59]. The balance between necrosis and apoptosis has implications for the potential immunomodulation that is induced by cryoablations.

This leads to the discussion of another mechanism of tumour destruction that purportedly occurs after cryoablation: stimulated immunological targeting of tumour cells. The observation that metastatic tumours sometimes regress after focal cryoablation of the primary tumour has been noted in many case reports and case series since this technique was first used to treat prostate cancer in the 1970s[60,61]. Early experimental data from rabbit and monkey models showed that organ-specific and tumour-specific serum antibodies were present after cryoablation[62–64]. As with RFA, the hypothesis is that the destruction of tumour tissue leaves intact tumour-specific antigens *in situ* that can stimulate an immune response against sublethally damaged or even untreated tissues[60].

However, cryoablation stands apart from the other modalities in that it induces a notably higher post-ablative immunogenicity[65]. After cryoablation, pro-inflammatory cytokines, including IL-1, IL-6 and nuclear factor-κB (NF-κB)-dependent cytokines such as TNFα, are released in higher quantities than after RFA and in even higher quantities than after MWA[32,33,66]. Markers of inflammation and hepatocyte injury — specifically, white blood cell count and liver transaminase levels, respectively — were significantly higher after cryoablation than after RFA or laser ablation in normal rat liver[65]. Antigen accumulation in DCs, as measured by magnetic bead sorting of labelled antigens, was also greater after cryoablation than after RFA of mice with melanomas[43]. In an innovative study that measured tumour gangliosides in the serum of patients with colon cancer who had hepatic metastases, cryoablation of the liver lesions resulted in a significant increase in serum ganglioside levels and an immunoglobulin M (IgM) titre that was specific to the ganglioside, but no such response occurred after RFA or surgical excision of the hepatic metastases[67].

A possible explanation for this is that high-temperature-based methods destroy tumours by a disruptive necrosis[68]: protein denaturation from heat reduces the amount of intact antitumour antigens, and heat coagulates tissue, thereby preventing the spill of intracellular products in large amounts into systemic circulation[65]. The freezing process, however, maintains intact intracytoplasmic organelles and the cell ultrastructure, while opening up the plasma membrane to immune cell exposure; this is in contrast to the unrecognizable,

amorphous intracytoplasmic contents that are seen using electron microscopy of radiofrequency-ablated normal rat liver[66].

A rare manifestation of this enhanced antitumour immunogenicity that is observed only after cryoablation of hepatocytes is known as cryoshock phenomenon. Cryoablation of hepatocytes results in the release of intracellular necrotic debris, which stimulates Kupffer cells to release pro-inflammatory mediators that can induce a systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulopathy, multi-system organ failure or death of either the animal or the patient[69]. This has been particularly noted when more than 35% of the liver volume is cryoablated[70]. SIRS does not occur when the hyperthermic modalities are used[33], which further supports the idea that RFA and MWA have a subdued systemic response compared with cryoablation.

The substantial response that is elicited by cryoablation has made it the target of extensive immunomodulatory research. Histological studies have shown post-cryoablation tumour infiltrates of neutrophils, followed by substantial macrophage recruitment; using enzyme-linked immunosorbent assays (ELISAs), freezing has also been shown to produce systemic elevation of tumour-specific antibodies[71]. NK cell activity[72], the tumour-specific T cell response in regional lymph nodes[72] and the level of systemic circulating T cells[65,68] have all been reported to increase after cryoablation in various models. Whether cryoablation stimulates a humoral response or cell-mediated immunity might depend on which monocytic mediator — DCs or macrophages — reaches the ablation site first or in larger quantities. DCs are particularly good at priming a T cell-mediated response, as they have a distinct ability to cross-present exogenous antigens on major histocompatibility complex (MHC) class I (which is integral to stimulating $CD8^+$ cytotoxic T lymphocytes (CTLs)). By contrast, macrophages cannot prime T cells. If macrophages are predominant in the post-cryoablated tissue, a humoral response might be more probable.

What further sets cryoablation apart in terms of immunomodulation is that it elicits not only an immunostimulatory effect but also a paradoxical immunosuppressive effect, as shown by many laboratory-based and clinical reports[31]. Experiments using animals have observed that the antitumour immune response was diminished when a larger amount of cryoablated tissue was left *in situ*[73] or when multiple liver nodules were cryoablated versus cryoablation of a single liver nodule[74]. The variable immune response to cryotherapy has been attributed to the balance between necrosis and apoptosis[31], both of which are processes of cell death that are seen during cryoablation. Necrosis results in an expulsion of intracellular contents (as discussed above): DNA, RNA, HSPs and HMGB1, which alert the innate immune system, macrophages and DCs[75,76]. By contrast, apoptosis, which is often a physiological occurrence, may induce immunosuppression towards the antigens of the cell. Large numbers of dying cells may repeatedly express self antigens without co-stimulatory danger signals, which results in peripheral tolerance. DCs that take up apoptotic cells without danger signals do not mature, have suppressed cytokine production, and may trigger clonal deletion and anergy[43,77].

Tumour cells have also been shown to release immunosuppressive cytokines, such as transforming growth factor-β (TGFβ) and IL-10. During cryoablation, if these cytokines are released instead of pro-inflammatory mediators, T_{Reg} cells may be stimulated to proliferate, and this also leads to immune tolerance. Increased numbers

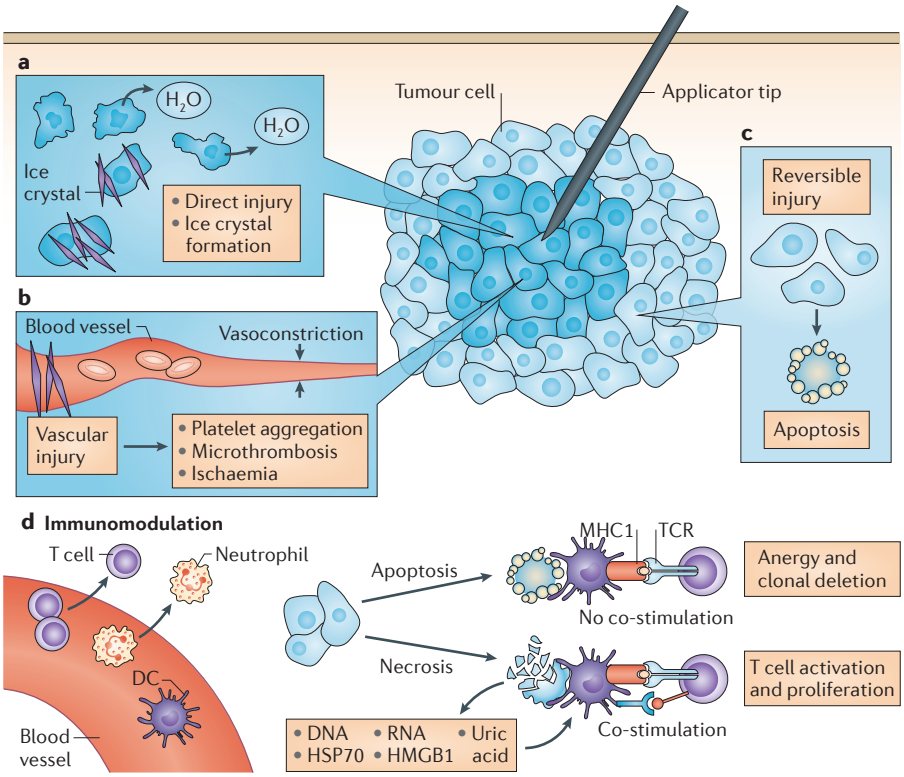


Figure 3 | **Mechanisms of cell death in cryoablation.** **a** | At the centre of the cryoablative lesion is a sharply demarcated area of frozen necrosis where direct injury occurs. Here, the temperature precipitously drops below –40 °C, and this causes ice to form from the extracellular space inwards. This results in a hypertonic extracellular environment and osmotic cell shrinkage from fluid shift out of the cell. The formation of ice crystals increases direct injury. **b** | Cold-induced vascular injury causes damage to endothelial cells and cell junctions, which leads to platelet aggregation and microthrombosis. Vasoconstriction occurs in response to cooling temperatures. Freezing also causes a hyperaemic response and increased vascular permeability. The resultant ischaemia causes further coagulative necrosis. **c** | Apoptosis occurs in a peripheral zone of sublethal cold temperatures, and this is probably induced by reversible damage. **d** | Blood vessels supply immune cell infiltrates. Both increased and reduced antitumour immunity can be induced by cryoablation; immunomodulation might depend on the predominant mode of cell death. Some tumour cells undergo apoptosis[14]. When antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages phagocytose tumour cells after apoptosis without danger signals, the tumour antigens are presented on major histocompatibility complex (MHC) class I molecules without co-stimulation of T cells. The dying cells can even secrete immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGFβ)[31]. This induces anergy and clonal deletion. Other tumour cells are necrotic, and they spill their extracellular contents: DNA, RNA, heat shock protein 70 (HSP70), uric acid and high mobility group protein B1 (HMGB1). Pro-inflammatory cytokines induce DCs to take up more antigen and express danger signals via co-stimulatory molecules that are necessary to prime nearby T cells. TCR, T cell receptor.

of regulatory or suppressive T cells have in fact been observed using animal models in studies that showed enhanced tumour metastases or inferior prognosis following cryoablation[78,79]. It is difficult to predict whether apoptosis or necrosis exerts more influence, and similarly, to predict the nature of the cytokines that are focally released — whether they are pro-inflammatory or immunosuppressive — and it has been suggested that these aspects are influenced by the method of ablation, the rate of freezing, the tumour type, age and individual differences in response[80]. Changes in the nature of the response over time, whether it is immunostimulatory or immunosuppressive, might also occur and result in sampling differences that are dependent on the time point at which a response is measured[80].

New and innovative areas of research

Understanding the biological factors that help or hinder thermal ablation has tremendous importance for enhancing or augmenting outcome. The intersections between energy-based ablations and tumour biology, immunology, chemotherapy or radiotherapy[18] are areas of rapidly expanding research. Combinatorial or synergistic therapies have the potential to improve survival, to decrease recurrence or metastasis and to lead to overall better targeted treatment of these tumours.

Overcoming the heat-sink effect. In the ablation modalities that rely on hyperthermia, blood or air flow carry heat away from the tissue, thereby decreasing the efficiency and substantially limiting the size of heat-mediated tumour destruction. RFA is notably susceptible to the heat-sink effect: even small tumours that are hypervascular are difficult targets. Several methods aim to decrease the blood flow near tumours to increase focal temperature; these include vascular clamping via surgery and arterial embolization with balloons, coils, particles or lipiodol agents. In randomized controlled trials and comparative studies[81,82] of patients with hepatocellular carcinoma, transarterial chemoembolization before RFA showed superior outcomes (overall survival[83] and disease recurrence rates[83,84]) compared with RFA alone.

Pharmacological agents that slow blood flow or are anti-angiogenic have been studied to help to decrease heat dissipation, while minimizing invasiveness. Administration of arsenic trioxide, which is a potent anti-vascular agent that has been used for leukaemia, significantly increased the size of RFA-induced coagulative necrosis in three animal models when it was used before ablation[19]. Furthermore, arsenic shows a dose-dependent synergy with RFA. How arsenic trioxide decreases tumour blood flow is not clearly understood, but the mechanism is thought to involve necrosis and thrombosis of tumour blood vessels. The disadvantage of arsenic trioxide is its high toxicity at the high doses that are necessary to achieve anti-vascular effects; further clinical investigations are needed to make this drug more clinically viable[85]. Halothane, which is known to decrease intrahepatic blood flow, has also been shown to significantly reduce focal tissue perfusion[86,87], which correlated with greater coagulative necrosis during RFA of normal porcine liver[86]. However, as a pharmacological agent, it has a limiting side effect profile that has thus far made it an unattractive agent for combination with ablation techniques.

The anti-angiogenic agent sorafenib has been studied in combination with RFA in animal studies[85]. Sorafenib is a vascular endothelial growth factor (VEGF) receptor and a platelet-derived growth factor (PDGF) receptor inhibitor that also inhibits the RAF kinase pathway in tumour endothelial cells. Like arsenic trioxide, sorafenib has been observed to markedly increase the RFA-induced area of coagulative necrosis, as shown in murine renal cell carcinoma models[85].

Synergy with immunotherapy. The immune response that is stimulated by energy-based ablations alone is often too modest to completely expunge established tumours[88]. Several investigations have studied strategies that combine immune adjuvants with thermal ablation to stimulate a more robust antitumour reaction, with the hope of a systemic immune response. Although these investigations are still in the preclinical stages, many have shown promising results (FIG. 4).

One approach is to follow cryoablation with administration of immunostimulants such as protein-bound polysaccharides[74] or toll-like receptor (TLR) activators in the form of unmethylated CpG motifs[89] or imiquimod[90,91]. In a murine model of intrasplenic injection of colon cancer to produce metastatic liver tumours, protein-conjugated polysaccharides were shown to suppress the production of IL-4 and IL-10, and thereby

Glossary

Acoustic energy
The energy that is generated by sound waves or oscillations in pressure.

Anergy
A form of T cell or B cell inactivation in which the cell remains alive but cannot be activated to execute an immune response. Anergy is a reversible state.

Brachytherapy
The implantation of radioactive pellets, which are approximately the size of a grain of rice, into the tissue that is being treated for cancer.

Clonal deletion
Elimination of T cells or B cells that have a high avidity for self antigens, either by negative selection during lymphocyte development or by FAS ligand-mediated destruction in the peripheral blood.

Coagulative necrosis
A form of tissue necrosis in which injury denatures structural proteins and enzymes, thereby prohibiting proteolysis of dead cells. Tissue architecture is preserved for days and necrotic debris is ultimately removed by infiltrating leukocytes.

Impedance
The effective resistance of an electric circuit.

Ischaemia
A reduced or lack of blood flow.

Lipiodol
Iodized poppyseed oil, which has been used for more than a century as a radiographic contrast agent.

Nitrosative stress
Inflammation and damage caused by reactive nitrogen species.

Pathogen-associated molecular pattern
(PAMP). A highly conserved structural motif that is commonly found on microorganisms. PAMPs include sugars, proteins, lipids and nucleic acids that are all recognized by the innate immune system.

Percutaneous
Pertaining to a procedure that is carried out through the skin.

Regulatory T cells
(T_{Reg} cells). A subset of T cells that display CD25 and that can inhibit $CD4^+$ and $CD8^+$ T cells. T_{Reg} cells express the transcription regulator forkhead box protein P3 (FOXP3), the lack of which predisposes to autoimmune diseases.

Three-dimensional radiotherapy
The application of radiation beams that are shaped to match the tumour to more precisely target it.

Transarterial chemoembolization
A procedure whereby chemotherapy is injected directly into the arterial supply of the tumour, and embolic agents are administered that cut off its blood supply.

Trocar-type probes
Surgical instruments with a three-sided cutting point enclosed in a hollow cylinder that is used to place other devices into the blood vessel or body cavity that it enters.

enhance the CTL and NK cell responses[74] that are directed towards the injected tumours.

TLRs are types of pattern-recognition receptors that are expressed on macrophages and DCs. When TLR9 and TLR7 are engaged by the pathogen-associated molecular pattern (PAMP) unmethylated CpG or the agonist imiquimod, respectively, they stimulate DCs, and this triggers T helper 1 cell (T_H1 cell) activation, which promotes CTL activity. Administration of both of these TLR agonists after cryoablation to attract DCs resulted in a more potent antitumour response in animal models.

In fact, harnessing DCs is an attractive way to induce a tumour-specific cell-mediated immunity. There are already clinical studies on the *ex vivo* loading of antigens onto mature DCs (also known as a conventional DC vaccination). Investigators are now also studying the injection of DCs following RFA or cryoablation in murine models. Antitumour immune activity can be increased by following thermal ablation with intratumoural injection of immature DCs[88]. In this case, tumour debris that is produced by thermal ablation functions as an effective *in situ* antigen source for DCs. Impressively, this approach induced an increased T_H1 response and tumour-specific CTLs, significantly prolonged survival and markedly reduced lung metastasis[88]. DC loading after cryoablation has been shown to be more effective than after RFA[43].

Another approach combines cryoablation with the blockade of cytotoxic T lymphocyte-associated antigen 4 (CTLA4)[43] to suppress the function of T_{Reg} cells. CTLA4, which is expressed on T_H1 cells and intracellularly in T_{Reg} cells, transmits an inhibitory signal to T cells. Generally, its expression is thought to restrain the antitumour activity of cell-mediated immunity. Ipilimumab is a US Food and Drug Administration (FDA)-approved CTLA4 antibody that may subvert immune system tolerance to tumours and thereby lead to a higher recognition and kill response. A preclinical proof-of-concept model of prostate cancer in mice showed that CTLA4 blockade in combination with cryoablation of the primary tumour could slow or prevent the growth of tumours that were challenged at a secondary site[92], whereas cryoablation alone could not significantly affect the growth of secondary tumours. The investigators also found that the tumours at challenge sites were highly infiltrated by $CD4^+$ and $CD8^+$ T cells and had a significantly higher ratio of effector T cells to T_{Reg} cells when compared with cryoablation alone.

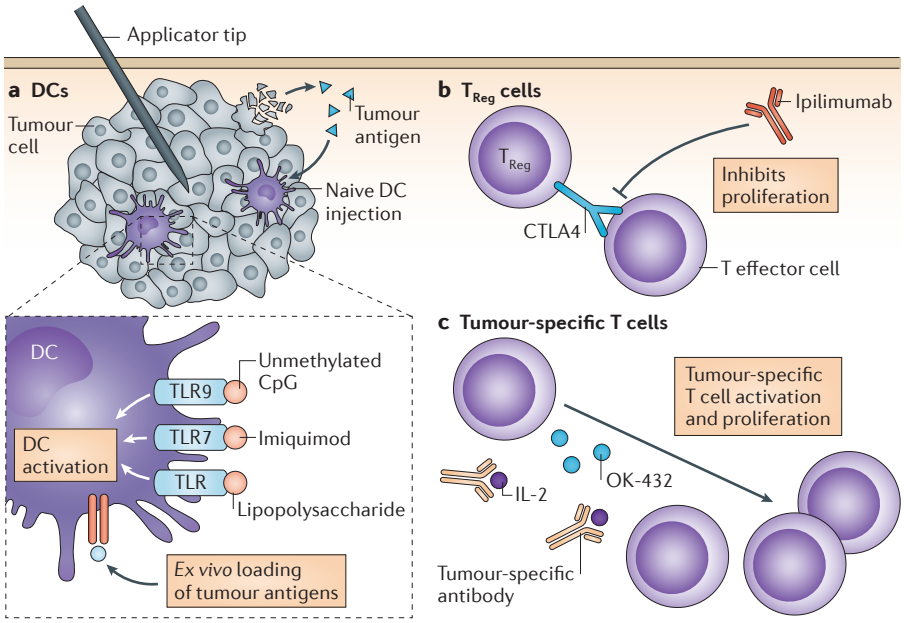


Figure 4 | **Potential immunotherapy targets that could be combined with ablation. a** | Dendritic cells (DCs) are a popular target for adjuvant immunotherapy in combination with ablation. DCs are professional antigen-presenting cells (APCs) and express both major histocompatibility complex (MHC) class I molecules, which present endogenous antigens, and MHC class II molecules, which present exogenous or foreign antigens. However, DCs can also cross-present exogenous antigens on MHC class I molecules, which is extremely important for activating a cytotoxic T cell response[31]. Several experimental techniques can target DCs. Toll-like receptors (TLRs) on DC membranes can be stimulated in various ways to activate DCs. For example, TLR9 can be can be stimulated using the pathogen-associated molecular pattern (PAMP) unmethylated CpG and TLR7 can be stimulated using the agonist imiquimod. Lipopolysaccharides (which are also PAMPs) can be used to activate DCs via TLR stimulation. In addition, *ex vivo*-generated DCs that are loaded with tumour antigens (from tumour cell lysate) can be re-injected during ablation[113]. Furthermore, naive DCs can be injected during ablation; this effectively produces an *in situ* tumour vaccine, with tumour antigens made available by ablation-induced necrosis[30]. **b** | Regulatory T cells (T_{Reg} cells) may promote immune system tolerance of tumour antigens. One of their expressed receptors is cytotoxic T lymphocyte-associated antigen 4 (CTLA4), which transmits an inhibitory signal to proliferating T lymphocytes and can downregulate the antitumour response. The blockade of CTLA4 using an inhibitory antibody (ipilimumab) can interfere with this immunosuppression against tumour antigens[92]. **c** | Tumour-specific T cells can be activated by different mechanisms. OK-432 is an immunostimulant that has been shown to enhance antitumour immunogenicity by activating T lymphocytes. The injection of OK-432 activates tumour-specific T cells and can cause tumour regression in animal models[114]. In addition, combining antitumour antibodies with interleukin-2 (IL-2) might increase antitumour T cell activity.[115]

Adoptive immunotherapy is the process of harvesting lymphocytes that have infiltrated metastatic tumours and expanding them *ex vivo* before restoring them in a greater quantity to the patient. One pioneering study of mammary adenocarcinoma in mice showed that adoptive transfer of tumour-specific T cells, which were increased in number after cryoablation and which were harvested from tumour-draining lymph nodes, resulted in a significant reduction in the number of pulmonary metastases when compared with adoptive transfer of the T cells harvested from tumour-draining lymph nodes after surgical excision or controls[93].

Synergy with conventional treatments. Combining thermal ablation with chemotherapy or radiotherapy can address one of the most important disadvantages of percutaneous ablation: incomplete or heterogeneous destruction.

The goal of adjuvant chemotherapy in thermal ablation is often to enhance tumour cell death in the peripheral or transitional zone, which, at sublethal temperatures, is an area recovering from reversible injury. Apoptosis that is triggered by heat-induced cell injury is increased by the cytotoxic injury of chemotherapies. One of the major roles of HSP70 is its protective effect against injury (as mentioned above)[40], and HSP70 has been shown to be particularly upregulated at the transitional zones of ablation[35,36].

Combining RFA with nanoparticle-delivered chemotherapies that enhance apoptosis or suppress HSPs has been shown to increase survival and tumour destruction in animal models[94]. In one study, RFA was combined with liposomal quercetin, which is a flavonoid that inhibits HSP expression; the co-administration resulted in an increased ablation zone compared with RFA alone[95].

Studies of rat breast tumour models have shown that combining intratumoural and intravascular doxorubicin with RFA can increase high-temperature-based coagulation[96,97]. A subsequent pilot clinical study of patients with hepatic tumours showed increased tumour destruction 2–4 weeks after ablation with combined liposomal doxorubicin and RFA, compared with RFA alone[98]. The strong synergistic effect has long been observed and has been attributed to cellular stress through the production of oxidative damage to DNA, nitrative damage to proteins and lipid injury, as well as activation and acceleration of apoptosis[37].

Liposomes have been used and studied as a chemotherapy delivery vehicle because of their predilection for the vasculature of solid tumours, which is pathologically leaky[99,100]. The peripheral or transitional zone of the ablation lesion is hyperaemic as a result of vasodilation and increased vascular permeability[101]; thus, it is well suited for increased delivery of liposomal chemotherapy. The induced hyperthermia increases endothelial pore size, which further improves access and deposition. Temperature-sensitive liposomes that release chemotherapeutic agents at elevated temperatures are a novel application of this research. Mild hyperthermia at 40 °C has been shown to increase the release and intracellular attachment of doxorubicin that is delivered in lyso-thermosensitive liposomes in human tumour xenograft models[102]. Targeted hyperthermia with RFA in combination with thermosensitive liposomal doxorubicin is now being tested in a randomized controlled trial as a novel approach for improving focal delivery and keeping systemic toxicity low[103]. Separately, empty liposomes combined with RFA showed increased cytotoxic effects, which suggests that liposomes themselves may improve coagulation by the generation of free radicals[98].

Combination radiotherapy and energy-based ablations in experimental animal studies have also shown increased tumour necrosis, reduced growth and overall improved survival compared with radiotherapy alone[104,105], as well as outcomes that are superior to combination RFA and liposomal doxorubicin[104]. These synergistic effects have been shown in novel clinical studies of patients with inoperable lung neoplasms, during both conventional three-dimensional radiotherapy[18] and brachytherapy[106]. One explanation for the synergy is that radiotherapy and thermal ablation have 'reciprocal zones of efficacy' (REF. 107). The highest risk of radiotherapy resistance occurs at the centre of the tumour because radiation depends on oxygen for its cytotoxicity and the centre of the tumour is the area that is most prone to hypoxia. In contradistinction, the margin of the tumour is where sublethal temperatures and reversible injury occur during thermal ablation, especially with the heat-sink effect. The hyperthermia, increased vasodilation and vascular permeability at the peripheral zone would increase the oxygenation in this area, which would further increase the efficacy of radiotherapy. Another possible mechanism for the synergistic effect is that radiotherapy impairs cell repair mechanisms and causes a persistence of free radicals. Markers of oxidative and nitrosative stress have been noted to be increased during combination therapy[2].

Percutaneous irreversible electroporation. Percutaneous IRE is one of the newest technologies in tumour ablation and does not actually use thermal energy. Instead, pulses of direct current that last from microseconds to milliseconds generate an electric field that causes irreversible cell damage, which leads to cell death[108]. Although the mechanism is not yet completely understood, the electric field is thought to change the electrochemical potential across the cell membrane, thereby causing instability of the lipid bilayer. The unstable membrane may cause structural changes, which can form aqueous pathways or nanoscale pores through the membrane[7]. Depending on the voltage, waveform and the frequency of the current, the electroporation pulse can also reversibly open the cell membrane, after which it repairs itself.

Reversible electroporation was therapeutically used before the development of IRE. It was introduced in the late 1980s[109] as electrochemotherapy (ECT), which was a technique designed to enhance chemotherapy delivery by making the tumour cell membrane more permeable. The combined effect of ECT and chemotherapy was found to be greater than either alone[7]. Compared to ECT, IRE is a less selective form of ablation, and in that way it is more similar to the other thermal techniques that are discussed above. Again compared to ECT, IRE has the advantage of not relying on toxic chemotherapeutic agents. One of its proposed advantages is its accuracy to the margin of tumours near other critical structures[7], which is a current challenge for the other thermoablation methods. In a histological study of the effects of IRE on pig liver, tissue ablation could be achieved to the margins of a lesion with an accuracy of the thickness of several cells in liver tissue[110]. Extracellular matrix, collagen structures and bile ducts remain intact because IRE selectively destroys lipid bilayers. In addition, because of the preservation of nearby vessels, rapid healing of the ablated tissue within 2 weeks was noted in the pig liver model[110].

IRE does generate heat close to the electrode, although this is not the dominant cytotoxic mechanism[111]. In fact, another major advantage of IRE compared to the other thermal modalities is that because IRE generally does not use high-temperature-based mechanisms, there is little to no heat-sink effect near vasculature, which is in contrast to RFA and MWA[112]. Furthermore, because the majority of the proteins in the electrical field are not denatured in IRE, the tumour antigens that are left in the ablated tissue theoretically remain intact. It will be interesting to see how IRE modulates the immune response during future investigations.

Future directions

Minimally invasive image-guided thermal ablation has become common since the advent of modern imaging. Most research in this field is currently still in the proof-of-principle, animal or preclinical phase. Whether this research can be generalized to humans is also limited by the heterogeneity of tumour types, animal models and ablation methodologies. There is a noticeable lack of randomized controlled clinical trials in patients, which is mostly because it is unethical to conduct head-to-head comparisons between ablation and surgical excision when the tumour is resectable. Nevertheless, impressive progress has been made in a matter of decades. Tumour size, for example, has long been an important restriction for the clinical use of thermal ablation, because incomplete ablation has led to lower local control rates. RFA, which was historically limited to lesions smaller than 3 cm, has benefited from recent advances in technology that have expanded its capability to up to 5 cm (REF. 1), which has consequently broadened its clinical indications.

From the simple ablation of small, unresectable tumours to experimental combinatorial therapies, percutaneous energy-based techniques have an expanding role in the treatment of solid neoplasms. Further elucidation of the complex antitumour

immune responses that are affected by cryoablation holds a lot of potential for adjunct immunotherapy. Measuring biomarkers such as cytokine profiles, HSP70 or serum antitumour antibody levels may provide prognostic information regarding survival and local control. Initial pilot clinical studies have already shown synergistic effects of ablation with chemotherapy or radiotherapy. There is clearly a great need for further translational investigation and clinical trials. Hopefully, the richness of the current research will continue to inform and guide this increasingly relevant field.

Katrina F. Chu and Damian E. Dupuy are at The Department of Diagnostic Imaging, The Warren Alpert Medical School of Brown University and Rhode Island Hospital, 593 Eddy Street, Providence, Rhode Island 02903, USA.
Correspondence to D.E.D.
e-mail: ddupuy@lifespan.org

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Competing interests statement
The authors declare competing interests: see Web version for details.