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Ultrasound-responsive cavitation nuclei for therapy and drug delivery

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ABSTRACT

Therapeutic ultrasound strategies are actively under development to harness the mechanical activity of cavitation nuclei for beneficial tissue bioeffects. The mechanical oscillations of circulating microbubbles, the most widely investigated cavitation nuclei, which may also encapsulate or shield a therapeutic agent in the bloodstream, trigger and promote localized uptake. Oscillating microbubbles can create stresses either on nearby tissue or in surrounding fluid to enhance drug penetration and efficacy in the brain, spinal cord, vasculature, immune system, biofilm, or tumors. This review summarizes recent investigations that have elucidated interactions of ultrasound and cavitation nuclei with cells, the treatment of tumors, immunotherapy, the blood brain barrier and blood spinal cord barrier, sonothrombolysis, cardiovascular drug delivery, and sonobactericide. In particular, an overview of salient ultrasound features, drug delivery vehicles, therapeutic transport routes, and preclinical and clinical studies is provided. Successful implementation of ultrasound and cavitation nuclei-mediated drug delivery has the potential to change the way drugs are administered systemically, resulting in more effective therapeutics and less-invasive treatments.

Key words: Ultrasound, Cavitation nuclei, Therapy, Drug delivery, Bubble-cell interaction, Sonoporation, Sonothrombolysis, Blood-brain barrier opening, Sonobactericide, Tumor.

INTRODUCTION

Around the start of the European Symposium on Ultrasound Contrast Agents (ESUCI), ultrasound-responsive cavitation nuclei were reported to have therapeutic potential. Thrombolysis was shown to be accelerated *in vitro* (Tachibana and Tachibana 1995) and cultured cells were transfected with plasmid DNA (Bao, et al. 1997). Since then, many research groups have investigated the use of cavitation nuclei for multiple forms of therapy, including both tissue ablation and drug and gene delivery. In the early years, the most widely investigated cavitation nuclei were gas microbubbles, ~1-10 µm in diameter and coated with a stabilizing shell, whereas nowadays both solid and liquid nuclei are also investigated that can be as small as a few hundred nm. Drugs can be co-administered with the cavitation nuclei or loaded in or on them (Lentacker, et al. 2009, Kooiman, et al. 2014). The diseases that can be treated with ultrasound-responsive cavitation nuclei include but are not limited to cardiovascular disease and cancer (Sutton, et al. 2013, Paefgen, et al. 2015), the current leading causes of death worldwide according to the World Health Organization (Nowbar, et al. 2019). This review focuses on the latest insights into cavitation nuclei for therapy and drug delivery from the physical and biological mechanisms of bubble-cell interaction to preclinical (both *in vitro* and *in vivo*) and clinical studies (timespan 2014-2019), with particular emphasis on the key clinical applications. The applications covered in this review are the treatment of tumors, immunotherapy, the blood brain barrier and blood spinal cord barrier, dissolution of clots, cardiovascular drug delivery, and the treatment of bacterial infections.

CAVITATION NUCLEI FOR THERAPY

The most widely used cavitation nuclei are phospholipid-coated microbubbles with a gas core. For the 128 preclinical studies included in the treatment sections of this review, the commercially available and clinically approved Definity® (Luminity® in Europe;

79 octafluoropropane gas core, phospholipid coating) (Definity® 2011, Nolsøe and Lorentzen
80 2016) microbubbles were used the most (in 22 studies). Definity® was used for studies on all
81 applications discussed here and the most for opening the blood brain barrier (BBB) (12
82 studies). SonoVue™ (Lumason® in the USA) is commercially available and clinically
83 approved as well (sulfur hexafluoride gas core, phospholipid coating) (Lumason® 2016, Nolsøe
84 and Lorentzen 2016) and was used in a total of 14 studies for the treatment of non-brain tumors
85 (for example Xing et al. (2016)), BBB opening (for example Goutal et al. (2018)), and
86 sonobactericide (for example Hu et al. (2018)). Other commercially available microbubbles
87 were used that are not clinically approved, such as BR38 (Schneider, et al. 2011) in the study
88 by Wang et al. (2015d) and MicroMarker (VisualSonics) in the study by Theek et al. (2016).
89 Custom-made microbubbles are as diverse as their applications, with special characteristics
90 tailored to enhance different therapeutic strategies. Different types of gasses were used as the
91 core such as air (for example Eggen et al. (2014)), nitrogen (for example Dixon et al. (2019)),
92 oxygen (for example Fix et al. (2018)), octafluoropropane (for example Pandit et al. (2019)),
93 perfluorobutane (for example Dewitte et al. (2015)), sulfur hexafluoride (Bae, et al. 2016,
94 Horsley, et al. 2019) or a mixture of gases such as nitric oxide and octafluoropropane (Sutton,
95 et al. 2014) or sulfur hexafluoride and oxygen (McEwan, et al. 2015). While fluorinated gases
96 improve the stability of phospholipid-coated microbubbles (Rossi, et al. 2011), other gases can
97 be loaded for therapeutic applications, such as oxygen to treat tumors (McEwan, et al. 2015,
98 Fix, et al. 2018, Nesbitt, et al. 2018) and nitric oxide (Kim, et al. 2014, Sutton, et al. 2014) or
99 hydrogen gas (He, et al. 2017) for treatment of cardiovascular disease. The main phospholipid
100 component of custom-made microbubbles is usually a phosphatidylcholine such as 1,2-
101 dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), used in 13 studies, for example Dewitte et
102 al. (2015), Bae et al. (2016), Chen et al. (2016), Fu et al. (2019), or 1,2-distearoyl-*sn*-glycero-
103 3-phosphocholine (DSPC), used in 18 studies, for example Kilroy et al. (2014), Bioley et al.

(2015), Dong et al. (2017), Goyal et al (2017), Pandit et al. (2019). These phospholipids are popular because they are also the main component in Definity® (Definity® 2011) and SonoVue®/Lumason® (Lumason® 2016), respectively. Another key component of the microbubble coating is a PEGylated emulsifier such as polyoxyethylene (40) stearate (PEG40-stearate; for example Kilroy et al. (2014)) or the most often used 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy (poly-ethyleneglycol) (DSPE-PEG2000; for example Belcik et al. (2017)), which is added to inhibit coalescence and to increase the *in vivo* half-life (Ferrara, et al. 2009). In general two methods are used to produce custom-made microbubbles: mechanical agitation (for example Ho et al. (2018)) or probe sonication (for example Belcik et al. (2015)). Both these methods produce a population of microbubbles that is polydisperse in size. Monodispersed microbubbles produced by microfluidics have recently been developed, and are starting to gain attention for pre-clinical therapeutic studies. Dixon et al. (2019) used monodisperse microbubbles to treat ischemic stroke.

Various therapeutic applications have inspired the development of novel cavitation nuclei, which is discussed in depth in the companion review by Stride et al. (2019). To improve drug delivery, therapeutics can be either co-administered with or loaded onto the microbubbles. One strategy for loading is to create microbubbles stabilized by drug-containing polymeric nanoparticles around a gas core (Snipstad, et al. 2017). Another strategy is to attach therapeutic molecules or liposomes to the outside of microbubbles, for example by biotin-avidin coupling (Dewitte, et al. 2015, McEwan, et al. 2016, Nesbitt, et al. 2018). Echogenic liposomes can be loaded with different therapeutics or gases and have been studied for vascular drug delivery (Sutton, et al. 2014), treatment of tumors (Choi, et al. 2014), and sonothrombolysis (Shekhar, et al. 2017). ACT® combines Sonazoid® microbubbles with droplets that can be loaded with therapeutics for treatment of tumors (Kotopoulis, et al. 2017). The cationic microbubbles utilized in the treatment sections of this review were used mostly for vascular drug delivery,

with genetic material loaded on the microbubble surface by charge-coupling (for example Cao et al. (2015)). Besides phospholipids and nanoparticles, microbubbles can also be coated with denatured proteins such as albumin. Optison™ (Optison™ 2012) is a commercially available and clinically approved ultrasound contrast agent that is coated with human albumin and used in studies on treatment of non-brain tumors (Xiao, et al. 2019), BBB opening (Kovacs, et al. 2017b, Payne, et al. 2017), and immunotherapy (Maria, et al. 2015). Nano-sized particles cited in this review have been used as cavitation nuclei for treatment of tumors, such as nanodroplets (for example Cao et al. (2018)) and nanocups (Myers, et al. 2016), for BBB opening (nanodroplets, Wu et al. (2018)), and for sonobactericide (nanodroplets, Guo et al. (2017a)).

BUBBLE-CELL INTERACTION

Physics

The physics of the interaction between bubbles or droplets and cells are described as these are the main cavitation nuclei used for drug delivery and therapy.

Physics of Microbubble – Cell Interaction

Being filled with gas and/or vapor makes bubbles highly responsive to changes in pressure and hence exposure to ultrasound can cause rapid and dramatic changes in their volume. These volume changes in turn give rise to an array of mechanical, thermal, and chemical phenomena that can significantly influence the bubbles' immediate environment and mediate therapeutic effects. For the sake of simplicity, these phenomena will be discussed in the context of a single bubble. It is important to note, however, that biological effects are typically produced by a population of bubbles and the influence of inter bubble interactions should not be neglected.

a. Mechanical effects

A bubble in a liquid is subject to multiple competing influences: the driving pressure of the imposed ultrasound field, the hydrostatic pressure imposed by the surrounding liquid, the

pressure of the gas and/or vapor inside the bubble, surface tension and the influence of any coating material, the inertia of the surrounding fluid, and damping due to the viscosity of the surrounding fluid and/or coating, thermal conduction, and/or acoustic radiation.

The motion of the bubble is primarily determined by the competition between the liquid inertia and the internal gas pressure. This competition can be characterized by using the Rayleigh-Plesset equation for bubble dynamics to compare the relative contributions of the terms describing inertia and pressure to the acceleration of the bubble wall (Flynn 1975a):

$$\ddot{R} = -\left(\frac{3}{2}\frac{\dot{R}^2}{R}\right) + \left(\frac{p_G(R)+p_\infty(t)-\frac{2\sigma}{R}}{\rho_L R}\right) = IF + PF, \qquad \text{(Eq. 1)}$$

where R is the time dependent bubble radius with initial value R_o, p_G is the pressure of the gas inside the bubble, p_∞ is the combined hydrostatic and time varying pressure in the liquid, σ is the surface tension at the gas liquid interface, and ρ_L is the liquid density.

Flynn (1975b, a) identified two scenarios: if the pressure factor (PF) is dominant when the bubble approaches its minimum size, then the bubble will undergo sustained volume oscillations. If the inertia term is dominant (IF), then the bubble will undergo inertial collapse, similar to an empty cavity, after which it may rebound or it may disintegrate. Which of these scenarios occurs is dependent upon the bubble expansion ratio: R_{max}/R_o, and hence the bubble size and the amplitude and frequency of the applied ultrasound field.

Both inertial and non-inertial bubble oscillations can give rise to multiple phenomena that impact the bubble's immediate environment and hence are important for therapy. These include:

(i) Direct impingement – even at moderate amplitudes of oscillation, the acceleration of the bubble wall may be sufficient to impose significant forces upon nearby surfaces, easily

deforming fragile structures such as a biological cell membranes (van Wamel, et al. 2006, Kudo 2017) or blood vessel walls (Chen, et al. 2011).

(ii) Ballistic motion – in addition to oscillating, the bubble may undergo translation as a result of the pressure gradient in the fluid generated by a propagating ultrasound wave (primary radiation force). Due to their high compressibility, bubbles may travel at significant velocities, sufficient to push them toward targets for improved local deposition of a drug (Dayton, et al. 1999) or penetrate biological tissue (Caskey, et al. 2009, Bader, et al. 2015, Acconcia, et al. 2016).

(iii) Microstreaming – when a structure oscillates in a viscous fluid there will be a transfer of momentum due to interfacial friction. Any asymmetry in the oscillation will result in a net motion of that fluid in the immediate vicinity of the structure known as microstreaming (Kolb and Nyborg 1956). This motion will in turn impose shear stresses upon any nearby surfaces as well as increasing convection within the fluid. Due to the inherently non-linear nature of bubble oscillations (equation 1), both non-inertial and inertial cavitation can produce significant microstreaming, resulting in fluid velocities on the order of 1 mm/s (Pereno and Stride 2018). If the bubble is close to a surface then it will also exhibit non-spherical oscillations which increases the asymmetry and hence the microstreaming even further (Nyborg 1958, Marmottant and Hilgenfeldt 2003).

(iv) Microjetting – another phenomenon associated with non-spherical bubble oscillations near a surface is the generation of a liquid jet during bubble collapse. If there is sufficient asymmetry in the acceleration of the fluid on either side of the collapsing bubble, then the more rapidly moving fluid may deform the bubble into a toroidal shape causing a high velocity jet to be emitted on the opposite side. Microjetting has been shown to be capable of producing pitting even in highly resilient materials such as steel (Naudé and Ellis 1961, Benjamin and Ellis 1966). However, as both the direction and velocity of the jet are determined by the elastic

properties of the nearby surface, its effects in biological tissue are more difficult to predict (Kudo and Kinoshita 2014). Nevertheless, as shown by Chen et al. (2011), in many cases a bubble will be sufficiently confined that microjetting will impact surrounding structures regardless of jet direction.

(v) Shockwaves – an inertially collapsing cavity that results in supersonic bubble wall velocities creates a significant discontinuity in the pressure in the surrounding liquid leading to the emission of a shockwave, which may impose significant stresses on nearby structures.

(vi) Secondary radiation force – at smaller amplitudes of oscillation a bubble will also generate a pressure wave in the surrounding fluid. If the bubble is adjacent to a surface, interaction between this wave and its reflection from the surface leads to a pressure gradient in the liquid and a secondary radiation force on the bubble. As with microjetting, the elastic properties of the boundary will determine the phase difference between the radiated and reflected waves and hence whether the bubbles move towards or away from the surface. Motion towards the surface may amplify the effects of (i), (iii), and (vi).

b. Thermal effects

As described above, an oscillating microbubble will reradiate energy from the incident ultrasound field in the form of a spherical pressure wave. In addition, the nonlinear character of the microbubble oscillations will lead to energy being reradiated over a range of frequencies. At moderate driving pressures the bubble spectrum will contain integer multiples (harmonics) of the driving frequency; and at higher pressures also fractional components (sub and ultraharmonics). In biological tissue, absorption of ultrasound increases with frequency and this nonlinear behavior thus also increases the rate of heating (Hilgenfeldt, et al. 2000, Holt and Roy 2001). Bubbles will also dissipate energy as a result of viscous friction in the liquid and thermal conduction from the gas core, the temperature of which increases during compression. Which mechanism is dominant depends on the size of the bubble, the driving

conditions and the viscosity of the medium. Thermal damping is however typically negligible in biomedical applications of ultrasound as the time constant associated with heat transfer is much longer than the period of the microbubble oscillations (Prosperetti 1977).

c. Chemical effects

The temperature rise produced in the surrounding tissue will be negligible compared with that occurring inside the bubble, especially during inertial collapse when it may reach several thousand Kelvin (Flint and Suslick 1991). The gas pressure similarly increases significantly. While only sustained for a very brief period, these extreme conditions can produce highly reactive chemical species, in particular reactive oxygen species (ROS), as well as the emission of electromagnetic radiation (sonoluminescence). ROS have been shown to play a significant role in multiple biological processes (Winterbourn 2008) and both ROS and sonoluminescence may affect drug activity (Rosenthal, et al. 2004, Trachootham, et al. 2009, Beguin, et al. 2019).

Physics of Droplets – Cell Interaction

Droplets consist of an encapsulated quantity of a volatile liquid, such as perfluorobutane (boiling point -1.7 °C) or perfluoropentane (boiling point 29 °C), which is in a superheated state at body temperature. Superheated state means that although the volatile liquids have a boiling point below 37 °C, these droplets remain in the liquid phase and do not show spontaneous vaporization after injection. Vaporization can be achieved instead by exposure to ultrasound of significant amplitude via a process known as acoustic droplet vaporization (ADV) (Kripfgans, et al. 2000). Before vaporization, the droplets are typically one order of magnitude smaller than the emerging bubbles, and the perfluorocarbon is inert and biocompatible (Biro and Blais 1987). These properties enable a range of therapeutic possibilities (Sheeran and Dayton 2012, Lea-Banks, et al. 2019). For example, unlike microbubbles, small droplets may extravasate from the leaky vessels into tumor tissue due to

253 the enhanced permeability and retention (EPR) effect (Long, et al. 1978, Lammers, et al. 2012,
254 Maeda 2012), and then be turned into bubbles by ADV (Rapoport, et al. 2009, Kopechek, et
255 al. 2013). Loading the droplets with a drug enables local delivery (Rapoport, et al. 2009) by
256 way of ADV. The mechanism behind this is that the emerging bubbles give rise to similar
257 radiation forces and microstreaming as described in the physics of the microbubble – cell
258 interaction above. It should be noted that oxygen is taken up during bubble growth
259 (Radhakrishnan, et al. 2016), which could lead to hypoxia.

260 The physics of the droplet – cell interaction is largely governed by the ADV. In general, it
261 has been observed that ADV is promoted by the following factors: large peak negative
262 pressures (Kripfgans, et al. 2000), usually obtained by strong focusing of the generated beam,
263 high frequency of the emitted wave, and a relatively long distance between the transducer and
264 the droplet. Another observation that has been made with micrometer-sized droplets is that
265 vaporization often starts at a well-defined nucleation spot near the side of the droplet where the
266 acoustic wave impinges (Shpak, et al. 2014). These facts can be explained by considering the
267 two mechanisms that play a role in achieving a large peak negative pressure inside the droplet:
268 acoustic focusing and nonlinear ultrasound propagation (Shpak, et al. 2016). In the following,
269 lengths and sizes are related to the wavelength, i.e. the distance traveled by a wave in one
270 oscillation (e.g., a 1 MHz ultrasound wave that is traveling in water with a wave speed, *c*, of
271 1500 m/s has a wavelength, *w* (m), of $\frac{c}{f} = \frac{1500}{10^6} = 0.0015$, *i.e.* 1.5 mm).

272 a. Acoustic focusing

273 Because the speed of sound in perfluorocarbon liquids is significantly lower than in water
274 or tissue, refraction of the incident wave will occur at the interface between these fluids, and
275 the spherical shape of the droplet will give rise to focusing. The assessment of this focusing
276 effect is not straightforward because the traditional way of describing these phenomena with
277 rays that propagate along straight lines (the ray approach) only holds for objects that are much

larger than the applied wavelength. In the current case, the frequency of a typical ultrasound wave used for insonification is in the order of 1-5 MHz, yielding wavelengths in the order of 1500 – 300 µm, while a droplet will be smaller by 2-4 orders of magnitude. Beside this, using the ray approach, the lower speed of sound in perfluorocarbon would yield a focal spot near the backside of the droplet, which is in contradiction to observations. The correct way to treat the focusing effect is to solve the full diffraction problem by decomposing the incident wave, the wave reflected by the droplet, and the wave transmitted into the droplet into a series of spherical waves. For each spherical wave, the spherical reflection and transmission coefficients can be derived. Superposition of all the spherical waves yields the pressure inside the droplet. Nevertheless, when this approach is only applied to an incident wave with the frequency that is emitted by the transducer, this will lead neither to the right nucleation spot nor to sufficient negative pressure for vaporization. Nanoscale droplets may be too small to make effective use of the focusing mechanism and ADV is therefore less dependent on the frequency.

b. Nonlinear ultrasound propagation

High pressure amplitudes, high frequencies, and long propagation distances all promote nonlinear propagation of an acoustic wave (Hamilton and Blackstock 2008). In the time domain, nonlinear propagation manifests itself as an increasing deformation of the shape of the ultrasound wave with distance traveled. In the frequency domain, this translates to increasing harmonic content, i.e. frequencies that are multiples of the driving frequency. The total incident acoustic pressure $p(t)$ at the position of a nanodroplet can therefore be written as

$$p(t) = \sum_{n=1}^{\infty} a_n \cos(n\omega t + \phi_n), \qquad \text{(Eq. 2)}$$

where which n is the number of a harmonic, a_n and ϕ_n are the amplitude and phase of this harmonic, and ω is the angular frequency of the emitted wave. The wavelength of a harmonic wave is a fraction of the emitted wavelength.

The above effects are both important in case of ADV and should therefore be combined. This implies that first the amplitudes and phases of the incident nonlinear ultrasound wave at the droplet location should be computed. Next, for each harmonic, the diffraction problem should be solved in terms of spherical harmonics. Adding the diffracted waves inside the droplet with the proper amplitude and phase will then yield the total pressure in the droplet. Figure 1 shows that the combined effects of nonlinear propagation and diffraction can cause a dramatic amplification of the peak negative pressure in the micrometer-sized droplet, sufficient for triggering droplet vaporization (Shpak, et al. 2014). Moreover, the location of the negative pressure peak also agrees with the observed nucleation spot.

After vaporization has started, the growth of the emerging bubble is limited by inertia and heat transfer. In the absence of the heat transfer limitation, the inertia of the fluid that surrounds the bubble limits the rate of bubble growth, which is linearly proportional to time and inversely proportional to the square root of the density of the surrounding fluid. When inertia is neglected, thermal diffusion is the limiting factor in the transport of heat to drive the endothermic vaporization process of perfluorocarbon, causing the radius of the bubble to increase with the square root of time. In reality, both processes occur simultaneously, where the inertia effect is dominant at the early stage and the diffusion effect is dominant at the later stage of bubble growth. The final size that is reached by a bubble depends on the time that a bubble can expand, i.e. on the duration of the negative cycle of the insonifying pressure wave. It is therefore expected that lower insonification frequencies give rise to larger maximum bubble size. Thus, irrespective of their influence on triggering ADV, lower frequencies would lead to more violent inertial cavitation effects and cause more biological damage, as experimentally observed for droplets with a radius in the order of 100 nm (Burgess and Porter 2019).

Biological mechanisms and bioeffects of ultrasound-activated cavitation nuclei

The biological phenomena of sonoporation (*i.e.* membrane pore formation), stimulated endocytosis, and opening of cell-cell contacts and the bioeffects of intracellular calcium transients, reactive oxygen species generation, cell membrane potential change, and cytoskeleton changes have been observed for several years (Sutton, et al. 2013, Kooiman, et al. 2014, Lentacker, et al. 2014, Qin, et al. 2018b). However, other bioeffects induced by ultrasound-activated cavitation nuclei have recently been discovered. These include membrane blebbing as a recovery mechanism for reversible sonoporation (both for ultrasound-activated microbubbles (Leow, et al. 2015) and upon ADV (Qin, et al. 2018a)), extracellular vesicle formation (Yuana, et al. 2017), suppression of efflux transporters P-glycoprotein (Cho, et al. 2016, Aryal, et al. 2017) and BBB (Blood Brain Barrier) transporter genes (McMahon, et al. 2018). At the same time, more insight has been gained in the origin of the bioeffects, largely through the use of live cell microscopy. For sonoporation, real time membrane pore opening and closure dynamics were revealed with pores <30 μm^2 closing within 1 min, while pores >100 μm^2 did not reseal (Hu, et al. 2013) as well as immediate rupture of filamentary actin at the pore location (Chen, et al. 2014) and correlation of intracellular reactive oxygen species levels with the degree of sonoporation (Jia, et al. 2018). Real-time sonoporation and opening of cell-cell contacts in the same endothelial cells has been demonstrated as well for a single example (Helfield, et al. 2016). The applied acoustic pressure was shown to determine uptake of model drugs via sonoporation or endocytosis in another study (De Cock, et al. 2015). Electron microscopy revealed formation of transient membrane disruptions and permanent membrane structures, *i.e.* caveolar endocytic vesicles, upon ultrasound and microbubble-treatment (Zeghimi, et al. 2015). A study by Fekri et al. (2016) revealed that enhanced clathrin-mediated endocytosis and fluid-phase endocytosis occur through distinct signaling mechanisms upon ultrasound and microbubble treatment. The majority of these bioeffects have

been observed in *in vitro* models using largely non-endothelial cells and may therefore not be directly relevant to *in vivo* tissue, where intravascular micron-sized cavitation nuclei will only have contact with endothelial cells and circulating blood cells. On the other hand, the mechanistic studies by Belcik et al. (2015, 2017) and Yu et al. (2017) do show translation from *in vitro* to *in vivo*. In these studies, ultrasound-activated microbubbles were shown to induce a shear-dependent increase in intravascular adenosine triphosphate (ATP) from both endothelial cells and erythrocytes, an increase in intramuscular nitric oxide, and downstream signaling through both nitric oxide and prostaglandins which resulted in augmentation of muscle blood flow. Ultrasound settings were similar, namely 1.3 MHz, MI 1.3 for Belcik et al. (2015, 2017) and 1 MHz, MI 1.5 for Yu et al. (2017), with MI defined as $MI = \frac{P_-}{\sqrt{f}}$ where P_- is the peak negative pressure of the ultrasound wave (in MPa) and *f* the center frequency of the ultrasound wave (in MHz).

Whether or not there is a direct relationship between the type of microbubble oscillation and specific bioeffects remains to be elucidated, although more insight has been gained through ultra-high-speed imaging of the microbubble behavior in conjunction with live cell microscopy. For example, there seems to be a microbubble excursion threshold above which sonoporation occurs (Helfield, et al. 2016). Van Rooij et al. (2016) further showed that displacement of targeted microbubbles enhanced reversible sonoporation and preserved cell viability whilst microbubbles that did not displace were identified as the main contributors to cell death.

All of the aforementioned biological observations, mechanisms, and effects relate to eukaryotic cells. Study of the biological effects of cavitation on for example bacteria is in its infancy, but studies suggest that sonoporation can be achieved in Gram– bacteria, with dextran uptake and gene transfection being reported in *Fusobacterium nucleatum* (Han, et al. 2007). More recent studies have investigated the effect of microbubbles and ultrasound on gene

expression (Li, et al. 2015, Dong, et al. 2017, Zhou, et al. 2018). The findings are conflicting because although they all show a reduction in expression of genes involved in biofilm formation and resistance to antibiotics, an increase in expression of genes involved with dispersion and detachment of biofilms was also found (Dong, et al. 2017). This cavitation-mediated bioeffect needs further investigation.

Modelling Microbubble – cell – drug interaction

Whilst there have been significant efforts to model the dynamics of ultrasound driven microbubbles (Faez, et al. 2013, Dollet, et al. 2019), less attention has been paid to the interactions between microbubbles and cells or their impact upon drug transport. Currently there are no models that describe the interactions between microbubbles, cells, and drug molecules. Several models have been proposed for the microbubble – cell interaction in sonoporation focusing on different aspects: the cell expansion and microbubble jet velocity (Guo, et al. 2017b), the shear stress exerted on the cell membrane (Wu 2002, Doinikov and Bouakaz 2010, Forbes and O'Brien 2012, Yu and Chen 2014, Cowley and McGinty 2019), microstreaming (Yu and Chen 2014), shear stress exerted on the cell membrane in combination with microstreaming (Li, et al. 2014), or other flow phenomena (Yu, et al. 2015, Rowlatt and Lind 2017) generated by an oscillating microbubble. In contrast to the other models, Man et al. (2019) propose that the microbubble-generated shear stress does not induce pore formation, but that this is instead due to microbubble fusion with the membrane and subsequent "pull out" of cell membrane lipid molecules by the oscillating microbubble. Models for pore formation (for example Koshiyama and Wada (2011)) and resealing (Zhang, et al. 2019) in cell membranes have also been developed, but these models neglect the mechanism by which the pore is created. There is just one sonoporation dynamics model, developed by Fan *et al*. (2012), that relates the uptake of the model drug propidium iodide (PI) to the size of the created

membrane pore and the pore resealing time for a single cell in an *in vitro* setting. The model describes the intracellular fluorescence intensity of PI as a function of time, *F(t)*, by:

$$F(t) = \alpha \cdot \pi D C_0 \cdot r_o \cdot \frac{1}{\beta}\left(1 - e^{-\beta t}\right), \quad \text{(Eq. 3)}$$

where α is the coefficient that relates the amount of PI molecules to the fluorescence intensity of PI-DNA and PI-RNA, D is the diffusion coefficient of PI, C_0 is the extracellular PI concentration, r_0 is the initial radius of the pore, β is the pore resealing coefficient, and t is time. The coefficient α is determined by the sensitivity of the fluorescence imaging system, and if unknown the equation can still be used because it is the pore size coefficient, $\alpha \cdot \pi D C_0 \cdot r_0$, that determines the initial slope of the PI uptake pattern and is the scaling factor for the exponential increase. A cell with a large pore will have a steep initial slope of PI uptake and the maximum PI intensity quickly reaches the plateau value. A limitation of this model is that equation 3 is based on two-dimensional free diffusion models, which holds for PI-RNA but not for PI-DNA because this is confined to the nucleus. The model is independent of cell type, as Fan et al. have demonstrated agreement with experimental results in both kidney (Fan, et al. 2012) and endothelial cells (Fan, et al. 2013). Other researchers have also used this model for endothelial cell studies and also classified the distribution of both the pore size and pore resealing coefficients using Principal Component Analysis to determine whether cells were reversibly or irreversibly sonoporated. In the context of blood brain barrier (BBB) opening, Hosseinkhah et al. (2015) have modeled the microbubble-generated shear and circumferential wall stress for 5 μm microvessels upon microbubble oscillation at a fixed mechanical index (MI) of 0.134 for a range of frequencies (0.5, 1, and 1.5 MHz). The wall stresses were dependent upon microbubble size (range investigated 2 – 18 μm in diameter) and ultrasound frequency. Wiedemair et al. (2017) have also modelled the wall shear stress generated by microbubble (2 μm diameter) destruction at 3 MHz for larger microvessels (200 μm diameter). The presence of red blood cells was included in the model and was found to cause confinement

of pressure and shear gradients to the vicinity of the microbubble. Advances in methods for imaging microbubble-cell interactions will facilitate the development of more sophisticated mechanistic models.

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TREATMENT OF TUMORS (NON-BRAIN)

The structure of tumor tissue varies significantly from that of healthy tissue which has important implications for its treatment. To support the continuous expansion of neoplastic cells, the formation of new vessels (i.e. angiogenesis) is needed (Junttila and de Sauvage 2013). As such, a rapidly-developed, poorly-organized vasculature with enlarged vascular openings arises. In between these vessels, large avascular regions exist, which are characterized by a dense extracellular matrix, high interstitial pressure, low pH, and hypoxia. Moreover, a local immunosuppressive environment is formed, preventing possible anti-tumor activity by the immune system.

Notwithstanding the growing knowledge of the pathophysiology of tumors, treatment remains challenging. Chemotherapeutic drugs are typically administered to abolish the rapidly-dividing cancer cells. Yet, their cytotoxic effects are not limited to cancer cells, causing dose-limiting off-target effects. To overcome this hurdle, chemotherapeutics are often encapsulated in nano-sized carriers, i.e. nanoparticles, that are designed to specifically diffuse through the large openings of tumor vasculature, while being excluded from healthy tissue by normal blood vessels (Lammers, et al. 2012, Maeda 2012). Despite being highly promising in pre-clinical studies, drug-containing nanoparticles have shown limited clinical success due to the vast heterogeneity in tumor vasculature (Barenholz 2012, Lammers, et al. 2012, Wang, et al. 2015d). In addition, drug penetration into the deeper layers of the tumor can be constrained due to high interstitial pressure and a dense extracellular matrix in the tumor. Furthermore,

acidic and hypoxic regions limit the efficacy of radiation- and chemotherapy-based treatments due to biochemical effects (Mehta, et al. 2012, McEwan, et al. 2015, Fix, et al. 2018). Ultrasound-triggered microbubbles are able to alter the tumor environment locally, thereby improving drug delivery to tumors. These alterations are schematically represented in Figure 2 and include: improving vascular permeability, modifying the tumor perfusion, reducing local hypoxia, and overcoming the high interstitial pressure.

Several studies have found that ultrasound-driven microbubbles improved delivery of chemotherapeutic agents in tumors, which resulted in increased anti-tumor effects (Wang, et al. 2015d, Snipstad, et al. 2017, Zhang, et al. 2018). Moreover, several gene products could be effectively delivered to tumor cells via ultrasound-driven microbubbles, resulting in a downregulation of tumor-specific pathways and an inhibition in tumor growth (Kopechek, et al. 2015, Zhou, et al. 2015). Theek et al. (2016) furthermore confirmed that nanoparticle accumulation can be achieved in tumors with low EPR effect. Drug transport and distribution through the dense tumor matrix and into regions with elevated interstitial pressure is often the limiting factor in peripheral tumors. As a result, several reports have indicated that drug penetration into the tumor remained limited after sonoporation, which may impede the eradication of the entire tumor tissue (Eggen, et al. 2014, Wang, et al. 2015d, Wei, et al. 2019). Alternatively, microbubble cavitation can affect tumor perfusion, as vasoconstriction and even temporary vascular shut-down have been reported *ex vivo* (Keravnou, et al. 2016) and *in vivo* (Hu, et al. 2012, Goertz 2015, Yemane, et al. 2018). These effects were seen at higher ultrasound intensities (>1.5 MPa) and are believed to result from inertial cavitation leading to violent microbubble collapses. As blood supply is needed to maintain tumor growth, vascular disruption might form a different approach to cease tumor development. Microbubble-induced microvascular damage was able to complement the direct effects of chemotherapeutics and anti-vascular drugs by secondary ischemia-mediated cytotoxicity, which led to tumor growth

inhibition (Wang, et al. 2015a, Ho, et al. 2018, Yang, et al. 2019b). In addition, a synergistic effect between radiation therapy and ultrasound-stimulated microbubble treatment was observed, as radiation therapy also induces secondary cell death by endothelial apoptosis and vascular damage (Lai, et al. 2016, Daecher, et al. 2017). Nevertheless, several adverse effects have been reported due to excessive vascular disruption, including hemorrhage, tissue necrosis, and the formation of thrombi (Goertz 2015, Wang, et al. 2015d, Snipstad, et al. 2017).

Furthermore, oxygen-containing microbubbles can provide a local oxygen supply to hypoxic areas, rendering oxygen-dependent treatments more effective. This is of interest for sonodynamic therapy, which is based on the production of cytotoxic reactive oxygen species (ROS) by a sonosensitizing agent upon activation by ultrasound in the presence of oxygen (McEwan, et al. 2015, McEwan, et al. 2016, Nesbitt, et al. 2018). As ultrasound can be used to stimulate the release of oxygen from oxygen-carrying microbubbles while simultaneously activating a sonosensitizer, this approach has shown to be particularly useful for the treatment of hypoxic tumor types (McEwan, et al. 2015, Nesbitt, et al. 2018). Additionally, low oxygenation promotes resistance to radiotherapy, which can be circumvented by a momentary supply of oxygen. Based on this notion, oxygen-carrying microbubbles were used to improve the outcome of radiotherapy in a rat fibrosarcoma model (Fix, et al. 2018).

Finally, ultrasound-activated microbubbles promote convection and induce acoustic radiation forces. As such, closer contact with the tumor endothelial and an extended contact time can be obtained (Kilroy, et al. 2014). Furthermore, these forces may counteract the elevated interstitial pressure present in tumors (Eggen, et al. 2014, Lea-Banks, et al. 2016, Xiao, et al. 2019).

Apart from their ability to improve the tumor uptake, microbubbles can be used as ultrasound-responsive drug carriers to reduce the off-target effects of chemotherapeutics. By loading the drugs or drug-containing nanoparticles directly in or onto the microbubbles, a

503 spatial and temporal control of drug release can be obtained, thereby reducing exposure to other
504 parts of the body (Yan, et al. 2013, Snipstad, et al. 2017). Moreover, several studies have shown
505 improved anti-cancer effects from treatment with drug-coupled microbubbles, compared to a
506 co-administration approach (Burke, et al. 2014, Snipstad, et al. 2017). Additionally, tumor
507 neovasculature expresses specific surface receptors that can be targeted by specific ligands.
508 Adding such targeting moieties to the surface of (drug-loaded) microbubbles improves site-
509 targeted delivery and has shown to potentiate this effect further (Bae, et al. 2016, Xing, et al.
510 2016, Luo, et al. 2017).

511 Phase-shifting droplets and gas-stabilizing solid agents (*e.g.* nanocups) have the unique
512 ability to benefit from both EPR-mediated accumulation in the 'leaky' parts of the tumor
513 vasculature due to their small sizes, as well as from ultrasound-induced permeabilization of the
514 tissue structure (Zhou 2015, Myers, et al. 2016, Liu, et al. 2018b, Zhang, et al. 2018). Several
515 research groups have reported tumor regression after treatment with acoustically-active
516 droplets (Gupta, et al. 2015, van Wamel, et al. 2016, Cao, et al. 2018, Liu, et al. 2018b) or gas-
517 stabilizing solid particles (Min, et al. 2016, Myers, et al. 2016). A different approach to the use
518 of droplets for tumor treatment, is Acoustic Cluster Therapy (ACT®), which is based on
519 microbubble-droplet clusters that upon ultrasound exposure, undergo a phase shift to create
520 large bubbles that can transiently block capillaries (Sontum, et al. 2015). While the mechanism
521 behind the technique is not yet fully understood, studies have shown improved delivery and
522 efficacy of paclitaxel and Abraxane® in xenograft prostate tumor models (van Wamel, et al.
523 2016, Kotopoulis, et al. 2017). Another use of droplets for tumor treatment is enhanced high-
524 intensity focused ultrasound (HIFU)-mediated heating of tumors (Kopechek, et al. 2014).

525 Although microbubble-based drug delivery to solid tumors shows great promise, it also
526 faces important challenges. The ultrasound parameters used in *in vivo* studies highly vary
527 between research groups and no consensus was found on the oscillation regime that is believed

to be responsible for the observed effects (Wang, et al. 2015d, Snipstad, et al. 2017). Moreover, longer ultrasound pulses and increased exposure times are usually applied in comparison to *in vitro* reports (Roovers, et al. 2019c). This could promote additional effects such as microbubble clustering and microbubble translation, which could cause local damage to the surrounding tissue as well (Roovers, et al. 2019a). To elucidate these effects further, fundamental *in vitro* research remains important. Therefore, novel *in vitro* models that more accurately mimic the complexity of the *in vivo* tumor environment are currently being explored. Park et al. (2016) engineered a perfusable vessel-on-a-chip system and reported successful doxorubicin delivery to the endothelial cells lining this microvascular network. While such microfluidic chips could be extremely useful to study the interactions of microbubbles with the endothelial cell barrier, special care to the material of the chambers should be taken to avoid ultrasound reflections and standing waves (Beekers, et al. 2018). Alternatively, 3D tumor spheroids have been used to study the effects of ultrasound and microbubble-assisted drug delivery on penetration and therapeutic effect in a multicellular tumor model (Roovers, et al. 2019b). Apart from expanding the knowledge on microbubble-tissue interactions in detailed parametric studies *in vitro*, it will be crucial to obtain improved control over the microbubble behavior *in vivo*, and link this to the therapeutic effects. To this end, passive cavitation detection (PCD) to monitor microbubble cavitation behavior in real-time is currently under development, and could provide better insights in the future (Choi, et al. 2014, Graham, et al. 2014, Haworth, et al. 2017). Efforts are being committed to constructing custom-built delivery systems, which can be equipped with multiple transducers allowing drug delivery guided by ultrasound imaging and/or PCD (Escoffre, et al. 2013, Choi, et al. 2014, Wang, et al. 2015c, Paris, et al. 2018).

Clinical studies

Pancreatic cancer

553 The safety and therapeutic potential of improved chemotherapeutic drug delivery using
554 microbubbles and ultrasound was first investigated for the treatment of inoperable pancreatic
555 ductal adenocarcinoma at Haukeland University Hospital, Norway (Kotopoulis, et al. 2013,
556 Dimcevski, et al. 2016). In this clinical trial, gemcitabine was administrated by intravenous
557 injection over 30 min. During the last 10 min of chemotherapy, an abdominal echography was
558 performed to locate the position of pancreatic tumor. At the end of chemotherapy, 0.5 mL of
559 SonoVue® microbubbles followed by 5 mL saline were intravenously injected every 3.5 min
560 to ensure their presence throughout the whole sonoporation treatment. Pancreatic tumors were
561 exposed to ultrasound (1.9 MHz, MI 0.2, 1% DC) using a 4C curvilinear probe (GE Healthcare)
562 connected to an LOGIQ 9 clinical ultrasound scanner. The cumulative ultrasound exposure
563 was only 18.9 s. All clinical data showed that microbubble-mediated gemcitabine delivery did
564 not induce any serious adverse events in comparison to chemotherapy alone. At the same time,
565 tumor size and development were characterized according to the Response Evaluation Criteria
566 in Solid Tumors (RECIST) criteria. In addition, Eastern Cooperative Oncology Group (ECOG)
567 performance status was used to monitor the therapeutic efficacy of the microbubble-mediated
568 gemcitabine delivery. All ten patients tolerated an increased number of gemcitabine cycles
569 compared to treatment with chemotherapy alone from historical controls (8.3 ± 6 *vs* 13.8 ± 5.6
570 cycles; $p < 0.008$), thus reflecting an improved physical state. After 12 treatment cycles, one
571 patient's tumor showed a 2-fold decrease in tumor size. This patient was excluded from this
572 clinical trial to be treated with radiotherapy and then with pancreatectomy. In five out of ten
573 patients, the maximum tumor diameter was partially decreased from the first to last therapeutic
574 treatment. Subsequently, a consolidative radiotherapy or a FOLFIRINOX treatment, a bolus
575 and infusion of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin, was offered to them. The
576 median survival was significantly increased from 8.9 months to 17.6 months ($p = 0.0001$).
577 Altogether, these results show that the drug delivery using clinically-approved microbubbles,

chemotherapeutics, and ultrasound is feasible and compatible with respect to clinical procedures. Nevertheless, the authors did not provide any evidence that the improved therapeutic efficacy of gemcitabine was related to an increase in intratumoral bioavailability of the drug. In addition, the effects of microbubble-assisted ultrasound treatment alone on the tumor growth were not investigated while recent publications describe that according to the ultrasound parameters, such treatment could induce a significant decrease in tumor volume through a reduction in tumor perfusion as described above.

Hepatic metastases from digestive system

A safety study of chemotherapeutic delivery using microbubble-assisted ultrasound for the treatment of liver metastases from gastrointestinal tumors and pancreatic carcinoma was conducted at Beijing Cancer Hospital, China (Wang, et al. 2018). Thirty minutes after intravenous infusion of chemotherapy (for both monotherapy and combination therapy), 1 mL of SonoVue® microbubbles was intravenously administrated which was repeated another five times in 20 min. An ultrasound probe (C1-5 abdominal convex probe; GE Healthcare, USA) was positioned on the tumor lesion which was exposed to ultrasound at different MIs (0.4 to 1) in contrast mode using a LogiQ E9 scanner (GE Healthcare, USA). The primary aims of this clinical trial were to evaluate the safety of this therapeutic procedure and to explore the largest MI and ultrasound treatment time which cancer patients can tolerate. According to the clinical safety evaluation, all twelve patients showed no serious adverse events. The authors reported that the microbubble mediated-chemotherapy led to fever in two patients. However, there is no clear evidence this related to the microbubble and ultrasound treatment. Indeed, in the absence of direct comparison of these results with a historical group of patients receiving the chemotherapy on its own, one cannot rule out a direct link between the fever and the chemotherapy alone. All the adverse side effects were resolved with symptomatic medication.

In addition, the severity of side effects did not worsen with increases in MI, suggesting that microbubble-mediated chemotherapy is a safe procedure. The secondary aims were to assess the efficacy of this therapeutic protocol using contrast-enhanced CT and MRI. Thus, tumor size and development were characterized according to the RECIST criteria. Half of the patients had stable disease and one patient obtained a partial response after the first treatment cycle. The median progression-free survival was 91 days. However, making any comparison and interpretation of results is very difficult because none of the patients were treated with the same chemotherapeutics, MI, and/or number of treatment cycles. The results of safety and efficacy evaluations should be compared to patients receiving the chemotherapy on its own in order to clearly identify the therapeutic benefit of combining with ultrasound-driven microbubbles. Similar to the pancreatic clinical study, no direct evidence of enhanced therapeutic bioavailability of the chemotherapeutic drug after the treatment was provided. This investigation is all the more important as the ultrasound and microbubble treatment was applied 30 min after intravenous chemotherapy (for both monotherapy and combination therapy) independently of drug pharmacokinetics and metabolism.

Ongoing and upcoming clinical trials

Currently, two clinical trials are ongoing: (i) Prof. F. Kiessling (RWTH Aachen University, Germany) proposes to examine whether the exposure of early primary breast cancer to microbubble-assisted ultrasound during neoadjuvant chemotherapy results in increased tumor regression in comparison to ultrasound treatment alone (NCT03385200); (ii) Dr. J. Eisenbrey (Sidney Kimmel Cancer Center, Thomas Jefferson University, USA) is investigating the therapeutic potential of perflutren protein-type A microspheres in combination with microbubble-assisted ultrasound in radioembolization therapy of liver cancer (NCT03199274).

A proof of concept study (NCT03458975) has been set in Tours Hospital, France for treating non-resectable liver metastases. The aim of this trial is to perform a feasibility study with the development of a dedicated ultrasound imaging and delivery probe with a therapy protocol optimized for patients with hepatic metastases of colorectal cancer and who are eligible for monoclonal antibodies in combination with chemotherapy. A dedicated 1.5D ultrasound probe has been developed and interconnected to a modified Aixplorer® imaging platform (Supersonic imagine, Aix-en-Provence, France). The primary objective of the study is to determine the rate of objective response at two months for lesions receiving optimized and targeted delivery of systemic chemotherapy combining bevacizumab and FOLFIRI compared with those treated with only systemic chemotherapy regimen. The secondary objective is to determine the safety and tolerability of this local approach of optimized intratumoral drug delivery during the three months of follow-up, by assessing tumor necrosis, tumor vascularity and pharmacokinetics of bevacizumab and by profiling cytokine expression spatially.

IMMUNOTHERAPY

Cancer immunotherapy is considered to be one of the most promising strategies to eradicate cancer as it makes use of the patient's own immune system to selectively attack and destroy tumor cells. It is a common name that refers to a variety of strategies that aim to unleash the power of the immune system by either boosting antitumoral immune responses or flagging tumor cells to make them more visible to the immune system. The principle is based on the fact that tumors express specific tumor antigens which are not, or to a much lesser extent, expressed by normal somatic cells and hence can be used to initiate a cancer-specific immune response. In this section we aim to give insight into how microbubbles and ultrasound have

651 been applied as useful tools to initiate or sustain different types of cancer immunotherapy as
652 illustrated in Figure 3.

653 When Ralph Steinman (Steinman, et al. 1979) discovered the dendritic cell (DC) in 1973,
654 its central role in the initiation of immunity made it an attractive target to evoke specific
655 antitumoral immune responses. Indeed, these cells very efficiently capture antigens and present
656 them to T-lymphocytes in major histocompatibility complexes (MHCs), thereby bridging the
657 innate and adaptive immune system. More specifically, exogenous antigens engulfed via the
658 endolysosomal pathway are largely presented to $CD4^+$ T cells *via* MHC-II, whereas
659 endogenous, cytoplasmic proteins are shuttled to MHC-I molecules for presentation to $CD8^+$
660 cells. As such, either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cell responses are induced. The
661 understanding of this pivotal role played by DCs formed the basis for DC-based vaccination,
662 where a patient's DCs are isolated, modified *ex vivo* to present tumor antigens and re-
663 administered as a cellular vaccine. DC-based therapeutics, however, suffer from a number of
664 challenges, of which the expensive and lengthy *ex vivo* procedure for antigen-loading and
665 activation of DCs is the most prominent (Santos and Butterfield 2018). In this regard,
666 microbubbles have been investigated for direct delivery of tumor antigens to immune cells *in*
667 *vivo*. Bioley et al. (2015) showed that intact microbubbles are rapidly phagocytosed by both
668 murine and human DCs, resulting in rapid and efficient uptake of surface-coupled antigens
669 without the use of ultrasound. Subcutaneous injection of microbubbles loaded with the model
670 antigen ovalbumin (OVA) resulted in the activation of both $CD8^+$ and $CD4^+$ T cells.
671 Effectively, these T-cell responses could partially protect vaccinated mice against an OVA-
672 expressing Listeria infection. Dewitte et al. (2014) investigated a different approach, making
673 use of messenger RNA (mRNA) loaded microbubbles combined with ultrasound to transfect
674 DCs. As such, they were able to deliver mRNA encoding both tumor antigens as well as
675 immunomodulating molecules directly to the cytoplasm of the DCs. As a result, preferential

presentation of antigen fragments in MHC-I complexes was ensured, favoring the induction of $CD8^+$ cytotoxic T cells. In a therapeutic vaccination study in mice bearing OVA-expressing tumors, injection of mRNA-sonoporated DCs caused a pronounced slowdown of tumor growth and induced complete tumor regression in 30% of the vaccinated animals. Interestingly, in humans, intradermally injected microbubbles have been used as sentinel lymph node detectors as they can easily drain from peripheral sites to the afferent lymph nodes (Sever, et al. 2012a, Sever, et al. 2012b). Since lymph nodes are the primary sites of immune induction, the interaction of microbubbles with intranodal DCs, could be of high value. To this end, Dewitte *et al.* (2015) showed that mRNA-loaded microbubbles were able to rapidly and efficiently migrate to the afferent lymph nodes after intradermal injection in healthy dogs. Unfortunately, further translation of this concept to an *in vivo* setting is not straightforward, as it prompts the use of less accessible large animal models (*e.g.*, pigs, dogs). Indeed, conversely to what has been reported in humans, lymphatic drainage of subcutaneously injected microbubbles is very limited in the small animal models typically used in preclinical research (mice and rats), which is the result of substantial difference in lymphatic physiology.

Another strategy in cancer immunotherapy is adoptive cell therapy, where *ex vivo* manipulated immune effector cells, mainly T cells and NK (natural killer) cells, are employed to generate a robust and selective anticancer immune response (Yee 2018, Hu, et al. 2019). These strategies have mainly led to successes in hematological malignancies, not only because of the availability of selective target antigens, but also because of the accessibility of the malignant cells (Khalil, et al. 2016, Yee 2018). By contrast, in solid tumors, and especially in brain cancers, inadequate homing of cytotoxic T cells or NK cells to the tumor proved to be one of the main reasons for the low success rates, making the degree of tumor infiltration an important factor in disease prognosis (Childs and Carlsten 2015, Gras Navarro, et al. 2015, Yee 2018). To address this, focused ultrasound and microbubbles have been used to make tumors

more accessible to cellular therapies. The first demonstration of this concept was provided by Alkins et al. (2013) who used a xenograft HER-2-expressing breast cancer brain metastasis model to determine whether ultrasound and microbubbles could allow intravenously infused NK cells to cross the blood-brain barrier (BBB). By loading the NK cells with superparamagnetic iron oxide (SPIO) nanoparticles, the accumulation of NK cells in the brain could be tracked and quantified via MRI. An enhanced accumulation of NK cells was found when the cells were injected immediately prior to BBB disruption. Importantly NK cells retained their activity and ultrasound treatment resulted in a sufficient NK to tumor cell ratio to allow effective tumor cell killing (Alkins, et al. 2016). In contrast, very few NK cells reached the tumor site when BBB disruption was absent or performed before NK cell infusion. Although it is not known for certain why timing had such a significant impact on NK extravasation, it is likely that the most effective transfer to the tissue occurs at the time of insonification, and that the barrier is most open during this time (Marty, et al. 2012). Possible other explanations include the difference in size of the temporal BBB openings or a possible alternation in the expression of specific leukocyte adhesion molecules by the BBB disruption, thus facilitating the translocation of NK cells. Also for tumors where BBB crossing is not an issue, ultrasound has been used to improve delivery of cellular therapeutics. Sta Maria et al. (2015) demonstrated enhanced tumor infiltration of adoptively transferred NK cells after treatment with microbubbles and low dose focused ultrasound. This result was confirmed by Yang *et al.* (2019a) in a more recent publication where the homing of NK cells was more than doubled after microbubble injection and ultrasound treatment of an ovarian tumor. Despite the enhanced accumulation, however, the authors did not observe an improved therapeutic effect, which might be due to the limited number of treatments that were applied, or the immunosuppressive tumor microenvironment that counteracts the cytotoxic action of the NK cells.

There is growing interest in exploring the effect of microbubbles and ultrasound on the tumor microenvironment, as recent work has shown that BBB disruption with microbubbles and ultrasound may induce sterile inflammation. Although a strong inflammatory response may be detrimental in the case of drug delivery across the BBB, it might be interesting to further study this inflammatory response in solid tumors as it might induce the release of damage-associated molecular patterns (DAMPS) such as heat-shock proteins and inflammatory cytokines. This could shift the balance towards a more inflammatory microenvironment that could promote immunotherapeutic approaches. As reported by Liu *et al.* (2012) exposure of a CT26 colon carcinoma xenograft to microbubbles and low pressure pulsed ultrasound increased cytokine release and triggered lymphocyte infiltration. Similar data have been reported by Hunt *et al.* (2015). In their study, ultrasound treatment caused a complete shut-down of tumor vasculature followed by the expression of HIF-1α (hypoxia-inducible factor 1α), a marker of tumor ischemia and tumor necrosis, as well as increased infiltration of T cells. Similar responses have been reported following thermal and mechanical HIFU treatments of solid tumors (Unga and Hashida 2014, Silvestrini, et al. 2017). A detailed review of ablative ultrasound therapies is however out of the scope of this review.

At present, the most successful form of immunotherapy is the administration of monoclonal antibodies to inhibit regulatory immune checkpoints that block T cell action. Examples are CTLA-4 (cytotoxic T lymphocyte-associated protein-4) and PD-1 (programmed cell death-1), which act as brakes on the immune system. Blocking the effect of these brakes can revive and support the function of immune effector cells. Despite the numerous successes achieved with checkpoint inhibitors, responses have been quite heterogeneous as the success of checkpoint inhibition therapy largely depends on the presence of intratumoral effector T cells (Weber 2017). This motivated Bulner et al. (2019) to explore the synergy of microbubble and ultrasound treatment with PD-L1 checkpoint inhibition therapy in mice. Tumors in the

751 treatment group that received the combination of microbubble and ultrasound treatment with
752 checkpoint inhibition were significantly smaller than tumors in the monotherapy groups. One
753 mouse showed complete tumor regression and remained tumor free upon rechallenge,
754 indicative of an adaptive immune response.

755 Overall, the number of studies that investigate the impact of microbubble and ultrasound
756 treatment on immunotherapy is limited, making this a rather unexplored research area. It is
757 obvious that more in-depth research is warranted to improve our understanding on how
758 (various types of) immunotherapy might benefit from (various types of) ultrasound treatment.

759

760 **BLOOD BRAIN BARRIER (BBB) AND BLOOD SPINAL CORD BARRIER (BSCB)**
761 **OPENING**

762 The barriers of the central nervous system (CNS), the Blood-Brain Barrier (BBB) and
763 Blood-Spinal Cord Barrier (BSCB), greatly limit drug-based treatment of CNS disorders.
764 These barriers help to regulate the specialized CNS environment by limiting the passage of
765 most therapeutically relevant molecules (Pardridge 2005). Although several methods have
766 been proposed to circumvent the BBB and BSCB, including chemical disruption and the
767 development of molecules engineered to capitalize on receptor-mediated transport (so-called
768 Trojan Horse molecules), the use of ultrasound in combination with microbubbles (Hynynen,
769 et al. 2001) or droplets (Wu, et al. 2018) to transiently modulate these barriers has come to the
770 forefront in recent years due to the targeted nature of this approach and its ability to facilitate
771 delivery of a wide range of currently available therapeutics. First demonstrated in 2001
772 (Hynynen, et al. 2001), ultrasound-mediated BBB opening has been the topic of several
773 hundred original research articles in the last two decades, and in recent years has made
774 headlines for ground-breaking clinical trials targeting brain tumors and Alzheimer's disease as
775 described below in the clinical studies section.

Mechanisms, Bioeffects, and Safety

Ultrasound in combination with microbubbles can produce permeability changes in the BBB via both enhanced paracellular and transcellular transport (Sheikov, et al. 2004, Sheikov, et al. 2006). Reduction and reorganization of tight junction proteins (Sheikov, et al. 2008) and upregulation of active transport protein Caveolin-1 (Deng, et al. 2012) have been reported. Although the exact physical mechanisms driving these changes are not known, there are several factors that are hypothesized to contribute to these effects, including direct tensile stresses due to the expansion and contraction of the bubbles in the lumen, as well as shear stresses at the vessel wall arising from acoustic microstreaming. Recent studies have also investigated the suppression of efflux transporters following ultrasound exposure with microbubbles. A reduction in P-glycoprotein expression (Cho, et al. 2016, Aryal, et al. 2017) and BBB transporter gene expression (McMahon, et al. 2018) has been observed by multiple groups. One study showed that P-glycoprotein expression was suppressed for over 48 h following treatment with ultrasound and microbubbles (Aryal, et al. 2017). However, the degree of inhibition of efflux transporters as a result of ultrasound with microbubbles may be insufficient to prevent efflux of some therapeutics (Goutal, et al. 2018), and thus this mechanism requires further study.

Many studies have documented enhanced CNS tumor response following ultrasound and microbubble-mediated delivery of drugs across the Blood-Tumor-Barrier in rodent models. Improved survival has been shown in both primary (Chen, et al. 2010, Aryal, et al. 2013) and metastatic tumor models (Park, et al. 2012, Alkins, et al. 2016).

Beyond simply enhancing drug accumulation in the CNS, several positive bioeffects of ultrasound and microbubble induced BBB opening have been reported. In rodent models of Alzheimer's disease, numerous positive effects have been discovered in the absence of

exogenous therapeutics. These effects include a reduction in amyloid-β plaque load (Jordão, et al. 2013, Burgess, et al. 2014, Leinenga and Götz 2015, Poon, et al. 2018), reduction in tau pathology (Pandit, et al. 2019), and improvements in spatial memory (Burgess, et al. 2014, Leinenga and Götz 2015). Two-photon microscopy has shown that amyloid-β plaque size is reduced in transgenic mice for up to two weeks post ultrasound and microbubble treatment (Poon, et al. 2018). Opening of the BBB in both transgenic and wild-type mice has also revealed enhanced neurogenesis (Burgess, et al. 2014, Scarcelli, et al. 2014, Mooney, et al. 2016) in the treated tissue.

Gene delivery to the CNS using ultrasound and microbubbles is another area that is increasingly being investigated. Viral (Alonso, et al. 2013, Wang, et al. 2015b) and non-viral (Mead, et al. 2016) delivery methods have been investigated. While early studies demonstrated the feasibility of gene delivery using reporter genes (for example Thevenot et al. (2012), Alonso et al. (2013)), there have been promising results delivering therapeutic genes. In particular, advances have been made in Parkinson's disease models, where therapeutic genes have been tested (Mead, et al. 2017, Xhima, et al. 2018), and where long lasting functional improvements have been reported in response to therapy (Mead, et al. 2017). It is expected that research into this highly promising technique will expand to a range of therapeutic applications.

Despite excellent safety profiles in non-human primate studies investigating repeat opening of the BBB (McDannold, et al. 2012, Downs, et al. 2015), there has been recent controversy due to reports of a sterile inflammatory response observed in rats (Kovacs, et al. 2017a, Kovacs, et al. 2017b, Silburt, et al. 2017). The inflammatory response is proportional to the magnitude of BBB opening and is therefore strongly influenced by experimental conditions such as microbubble dose and acoustic settings. However, McMahon and Hynynen (2017) showed that when clinical microbubble doses are used, and treatment exposures are actively controlled to avoid over treating, the inflammatory response is acute and mild. They note that while chronic

inflammation is undesirable, acute inflammation may actually contribute to some of the positive bioeffects that have been observed. For example, the clearance of amyloid-β following ultrasound and microbubble treatment is thought to be mediated in part by microglial activation (Jordão, et al. 2013). These findings reiterate the need for carefully controlled treatment exposures to select for desired bioeffects.

Cavitation Monitoring and Control

It is generally accepted that the behavior of the microbubbles in the ultrasound field is predictive, to an extent, of the observed bioeffects. In the seminal study on the association between cavitation and BBB opening, McDannold et al. (2006) observed an increase in second harmonic emissions in cases of successful opening, compared to exposures that lead to no observable changes in permeability as measured by contrast enhanced MRI. Further, they noted that successful opening could be achieved in the absence of inertial cavitation, which was also reported by another group (Tung, et al. 2010). These general guidelines have been central to the development of active treatment control schemes that have been developed to date – all with the common goal of promoting stable bubble oscillations, while avoiding violent bubble collapse that can lead to tissue damage. These methods are based either on detection of sub or ultraharmonic (O'Reilly and Hynynen 2012, Tsai, et al. 2016, Bing, et al. 2018), harmonic bubble emissions (Arvanitis, et al. 2012, Sun, et al. 2017) or a combination thereof (Kamimura, et al. 2019). An approach based on the sub/ultraharmonic controller developed by O'Reilly and Hynynen (2012) has been employed in early clinical testing (Lipsman, et al. 2018, Mainprize, et al. 2019).

Control methods presented to date have generally been developed using single receiver elements, which simplifies data processing but does not allow signals to be localized. Focused receivers are spatially selective but can miss off-target events, while planar receivers may

generate false positives based on signals originating outside the treatment volume. The solution to this is to use an array of receivers and passive beamforming methods, combined with phase correction methods to compensate for the skull bone (Jones, et al. 2013, 2015) to generate maps of bubble activity. In the brain this has been achieved with linear arrays (Arvanitis, et al. 2013, Yang, et al. 2019c), which suffer from poor axial resolution when using passive imaging methods, as well as large-scale sparse hemispherical or large aperture receiver arrays (O'Reilly, et al. 2014, Deng, et al. 2016, Crake, et al. 2018, Jones, et al. 2018, Liu, et al. 2018a) that optimize spatial resolution for a given frequency. Recently, this has extended beyond just imaging the bubble activity to incorporate real-time, active feedback control based on both the spectral and spatial information obtained from the bubble maps (Jones, et al. 2018) (Figure 4). Robust control methods building on these works will be essential for widespread adoption of this technology to ensure safe and consistent treatments.

BSCB opening

Despite the similarities between the BBB and BSCB, and the great potential benefit for patients, there has been limited work investigating translation of this technology to the spinal cord. Opening of the BSCB in rats was first reported by Wachsmuth et al. (2009), and was followed by studies from Weber-Adrien et al. (2015), Payne et al. (2017), and O'Reilly et al. (2018) in rats (Figure 5) and from Montero et al. (2019) in rabbits, the latter performed through a laminectomy window. In 2018, O'Reilly et al. (2018) presented the first evidence of a therapeutic benefit in a disease model, showing improved tumor control in a rat model of leptomeningeal metastases.

Although promising, there remains significant work to be done to advance BSCB opening to clinical studies. A more thorough characterization of the bioeffects in the spinal cord and how, if at all, they differ from the brain is necessary to ensure safe translation. Additionally,

methods and devices capable of delivering controlled therapy to the spinal cord at clinical scale are needed. While laminectomy and implantation of an ultrasound device (Montero, et al. 2019) might be an appropriate approach for some focal indications, treating multifocal or diffuse disease will require the ultrasound to be delivered through the intact bone to the narrow spinal canal. Fletcher and O'Reilly (2018) have presented a method to suppress standing waves in the human vertebral canal. Combined with devices suited to the spinal geometry, such as that presented by Xu and O'Reilly (2019), these methods will help to advance clinical translation.

Clinical studies

The feasibility of enhancing BBB permeability in and around brain tumors using ultrasound and microbubbles has now been demonstrated in two clinical trials. In the study conducted at Assistance Publique–Hôpitaux de Paris in Paris, France, an unfocused 1 MHz ultrasound transducer (SonoCloud®) was surgically placed over the tumor-resection area and permanently fixed into the hole in the skull bone. The skin was placed over the transducer and after healing, treatments were conducted by inserting a needle probe through the skin to provide the driving signal to the transducer. Monthly treatments were then conducted while infusing a chemotherapeutic agent into the blood stream (carboplatin). The sonication was executed during infusion of SonoVue® microbubbles. A constant pulsed sonication was applied during each treatment followed by a contrast enhanced MRI to estimate BBB permeability. The power was escalated for each monthly treatment until enhancement was detected in MRI. This study demonstrated feasibility and safety (Carpentier, et al. 2016) and a follow up study may indicate increase in survival (Idbaih, et al. 2019).

The second brain tumor study was conducted at Sunnybrook Health Sciences Centre in Toronto, Canada, which used the InSightec Exablate 220 kHz device and through-skull MRI–guided sonications of brain tumors prior to the surgical resection. It also showed the feasibility

of inducing highly localized BBB permeability enhancement, safety, and that chemotherapeutic concentration in the sonicated peritumor tissue was higher than in the unsonicated tissue (Mainprize, et al. 2019).

Another study conducted in Alzheimer's disease patients with the Exablate device demonstrated safe BBB permeability enhancement and that the treatment could be repeated one month later without any imaging or behavior indications of adverse events (Lipsman, et al. 2018). A third study with the same device investigated the feasibility of using functional MRI to target motor cortex in Amyotrophic Lateral Sclerosis (ALS) patients again showing precisely targeted BBB permeability enhancement without adverse effects in this delicate structure (Abrahao, et al. 2019). All of these studies were conducted using Definity® microbubbles. These studies have led to the current ongoing brain tumor trial with six monthly treatments of the brain tissue surrounding the resection cavity during the maintenance phase of the treatment with temozolomide. This study sponsored by InSightec is being conducted in multiple institutions. Similarly, a phase II trial in Alzheimer's disease sonicating the hippocampus with the goal of investigating the safety and potential benefits from repeated (three treatments with two-week interval) BBB permeability enhancement alone is ongoing. This study is also being conducted in several institutions that have the device.

SONOTHROMBOLYSIS

Occlusion of blood flow through diseased vasculature is caused by thrombi, blood clots which form in the body. Due to limitations in thrombolytic efficacy and speed, sonothrombolysis, ultrasound which accelerates thrombus breakdown alone, or in combination with thrombolytic drugs and/or cavitation nuclei, has been under extensive investigation in the last two decades (Bader, et al. 2016). Sonothrombolysis promotes thrombus dissolution for the treatment of stroke (Alexandrov, et al. 2004a, Alexandrov, et al. 2004b, Molina, et al. 2006,

Chen, et al. 2019), myocardial infarction (Mathias, et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019), acute peripheral arterial occlusion (Ebben, et al. 2017), deep vein thrombosis (Shi, et al. 2018), and pulmonary embolism (Dumantepe, et al. 2014, Engelberger and Kucher 2014, Lee, et al. 2017).

Mechanisms, Agents, and Approaches

Ultrasound improves recombinant tissue plasminogen activator (rt-PA) diffusion into thrombi and augments lysis primarily via acoustic radiation force and streaming (Datta, et al. 2006, Prokop, et al. 2007, Petit, et al. 2015). Additionally, ultrasound increases rt-PA and plasminogen penetration into the thrombus surface and enhances removal of fibrin degradation products via ultrasonic bubble activity, or acoustic cavitation, that induces microstreaming (Elder 1958, Datta, et al. 2006, Sutton, et al. 2013). Two types of cavitation are correlated with enhanced thrombolysis: stable cavitation, with highly nonlinear bubble motion resulting in acoustic emissions at the subharmonic and ultraharmonics of the fundamental frequency (Flynn 1964, Phelps and Leighton 1997, Bader and Holland 2013), and inertial cavitation, with substantial radial bubble growth and rapid collapse generating broadband acoustic emissions (Carstensen and Flynn 1982, Flynn 1982).

Specialized contrast agents and tailored ultrasound schemes have been investigated with the aim of optimizing sonothrombolysis. Petit et al. (2015) observed a greater degree of rt-PA lysis with BR38 microbubbles exposed to 1 MHz pulsed ultrasound at an amplitude causing inertial cavitation (1.3 MPa peak rarefactional pressure) than at a lower amplitude causing stable cavitation (0.35 MPa peak rarefactional pressure). Goyal et al. (2017) also measured a higher degree of thrombolysis with 1 MHz pulsed ultrasound at 1.0 MPa peak rarefactional pressure with inertial cavitation than at 0.23 MPa peak rarefactional pressure with stable cavitation in an *in vitro* model of microvascular obstruction using perfluorobutane-filled, lipid

shelled microbubbles (Weller, et al. 2002) as a nucleation agent. However, Kleven et al. (2019) observed more than 60% fractional clot width loss for highly retracted human whole blood clots exposed to rt-PA, Definity® and 220 kHz pulsed or continuous wave (CW) ultrasound at an acoustic output with sustained stable cavitation throughout the insonification periods (0.22 MPa peak rarefactional pressure) (Figure 6).

Echogenic liposomes loaded with rt-PA enhanced lysis compared to rt-PA alone at concentrations of 1.58 and 3.15 mg/mL (Shekhar, et al. 2017), suggesting that encapsulation of rt-PA could reduce the rt-PA dose by a factor of two with equivalent lytic activity. Subsequently it has been demonstrated that these liposomes protect rt-PA against degradation by plasminogen activator inhibitor-1 (PAI-1), while achieving equivalent thrombolytic efficacy relative to rt-PA, Definity®, and intermittent 220 kHz CW ultrasound (Shekhar, et al. 2019). Promising agents, including a nanoscale (< 100 nm) contrast agent (Brüssler, et al. 2018) and magnetically targeted microbubbles (De Saint Victor, et al. 2019), have also demonstrated enhanced rt-PA thrombolysis *in vitro*. All of these investigators noted that in the absence of rt-PA, the combination of ultrasound and microbubbles did not degrade the fibrin network.

Several minimally invasive techniques have also been explored, with or without the inclusion of rt-PA or exogenous cavitation nuclei. In the clinical management of stroke, rapid treatments are needed because of the neurologist's adage "time is brain". Thus, treatment options that promote fast clot removal, reduce edema and intracerebral bleeding, and improve patient outcomes are of immense value. Magnetic resonance image-guided high intensity focused ultrasound has been investigated for the treatment of both ischemic (Burgess, et al. 2012) and hemorrhagic (Monteith, et al. 2013) stroke, and Zafar et al. (2019) have provided an excellent review of the literature for this approach. Histotripsy, a form of high intensity focused ultrasound that relies on the mechanical action of microbubble clouds to ablate thrombi with

and without rt-PA (Maxwell, et al. 2009, Bader, et al. 2015, Zhang, et al. 2016b, Bader, et al. 2019) is under development to treat deep vein thrombosis. Additionally, ultrasound-accelerated catheter-directed thrombolysis using the EKOS system (EKOS/BTG, Bothell, WA, USA) combines 2 MHz low-intensity pulsed ultrasound and rt-PA without cavitation nuclei to improve lytic efficiency to treat deep vein thrombosis (Shi, et al. 2018) and pulmonary embolism (Garcia 2015).

Cavitation monitoring

Acoustic cavitation has been shown to mediate direct fibrinolysis (Weiss, et al. 2013) and accelerated rt-PA lysis (Everbach and Francis 2000, Datta, et al. 2006, Prokop, et al. 2007, Hitchcock, et al. 2011). Passive and active cavitation detection techniques have been developed to monitor acoustic cavitation (Roy, et al. 1990, Madanshetty, et al. 1991, Bader, et al. 2015). Passive cavitation imaging, or passive acoustic mapping, employs a transducer array that listens passively (i.e., no transmit) to emissions from acoustically activated microbubbles (Salgaonkar, et al. 2009, Gyöngy and Coussios 2010, Haworth, et al. 2017). Vignon et al. (2013) developed a prototype array enabling spectral analysis of bubble activity for sonothrombolysis applications. Superharmonic Doppler effects have also been utilized to monitor bubble activity from 500 kHz pulsed therapeutic ultrasound (Pouliopoulos and Choi 2016). Both a linear array (Arvanitis and McDannold 2013a, Arvanitis, et al. 2013, Arvanitis and McDannold 2013b) and a sparse hemispherical array (Acconcia, et al. 2017) have been integrated into a clinical magnetic resonance image-guided high intensity focused ultrasound system to assess microbubble dynamics during sonothrombolysis in the brain.

Preclinical studies

Information gathered from animal studies can help inform human clinical trials, despite a strong species dependence of clot rt-PA lytic susceptibility (Gabriel, et al. 1992, Flight, et al. 2006, Huang, et al. 2017). A comprehensive systematic evaluation of 16 *in vivo* preclinical sonothrombolysis studies was carried out by Auboire et al. (2018) summarizing treatment efficacy and safety outcomes in models of ischemic stroke. Since that review was published, the efficacy of sonothrombolysis using nitrogen microbubbles stabilized with a non-crosslinked shell delivered intra-arterially through a catheter and rt-PA delivered intravenously has been demonstrated in a rat model of ischemic stroke (Dixon, et al. 2019).

Clinical studies

A rich literature exists of clinical trials exploring the safety and efficacy of sonothrombolysis. Two recent meta-analyses of seven randomized controlled trials (Chen, et al. 2019, Zafar, et al. 2019) attempt to determine whether the administration of rt-PA and ultrasound improve outcomes in acute ischemic stroke. Both analyses conclude that sonothrombolysis significantly enhances complete or partial recanalization, with improved neurologic function (assessed via the National Institutes of Health Stroke Scale, NIHSS). An ongoing clinical trial (TRUST; NCT03519737) will determine whether large vessel occlusions can be recanalized with sonothrombolysis (Cerevast Medical, Inc., Bothell, WA, USA) and rt-PA, tenecteplase or alteplase, (Campbell, et al. 2018) while patients are transferred to a stroke center for mechanical thrombectomy (Gauberti 2019).

Several clinical trials have shown that high MI pulsed diagnostic ultrasound exposure of Definity® before and after percutaneous coronary intervention for ST elevation myocardial infarction can prevent microvascular obstruction and improve functional outcomes (Mathias, et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019). A systematic review of 16 catheter-directed sonothrombolysis clinical trials comprised mostly of retrospective case series using

the EKOS system without microbubble infusions determined that this treatment modality is safe and promising for the treatment of deep vein thrombosis, DVT (Shi, et al. 2018). However, a large-sample randomized prospective clinical trial is needed to improve the clinical evidence for use as a front-line therapy for DVT. In retrospective studies in patients with pulmonary embolism Lee et al. (2017) conclude that catheter directed sonothrombolysis is safe and decreases right-sided heart strain, but Schissler et al. (2018) conclude that this therapy is not associated with a reduction in mortality nor increased resolution of right ventricular dysfunction. And finally, an ongoing trial in a small cohort of 20 patients with acute peripheral arterial occlusions (Ebben, et al. 2017) will determine whether Luminity® (marketed in the US as Definity®) and 1.8 MHz transdermal diagnostic ultrasound with intermittent high MI (1.08) and low MI (0.11) for visualization of the microbubbles and flow will improve recanalization. In summary, sonothrombolysis has demonstrated clinical benefit in the treatment of acute and chronic thrombotic disease. Ultrasound-assisted thrombolysis has a potential role as an emerging viable and therapeutic option for future management of stroke and cardiovascular disease.

CARDIOVASCULAR DRUG DELIVERY AND THERAPY

In cardiovascular drug delivery, cavitation nuclei are co-administered or loaded with different therapeutics for the treatment of various diseases. For atherosclerosis treatment in an ApoE-deficient mouse model, intercellular adhesion molecule-1 targeted microbubbles carrying angiogenesis inhibitor Endostar were used (Yuan, et al. 2018). Upon intermittent insonification over the abdominal and thoracic cavity with 1 MHz ultrasound (2 W/cm^2 intensity, 50% duty cycle) for 30 s with two repeats and another treatment 48 h later, plaque area and intraplaque neovascularization were significantly reduced two weeks after treatment. Percutaneous coronary intervention is often used to restore blood flow in atherosclerotic

arteries. The treatment of coronary microembolization, a complication of percutaneous coronary intervention, was demonstrated in pigs treated with ultrasound (1 MHz, 2.0 W/cm^2 intensity, 10 s on and 10 s off, 20 min duration) and microRNA-21-loaded microbubbles four days before coronary microembolization (Su, et al. 2015). This resulted in an improved cardiac dysfunction. Although not a therapeutic study, Liu et al. (2015) did show that plasmid transfection to the myocardium was significantly larger when the microbubbles were administered into the coronary artery compared to intravenously via the ear vein in pigs even though the intracoronary microbubble dose was half of the intravenous dose (1 MHz ultrasound, 2 W/cm^2, 50% duty cycle, 20 min duration). Percutaneous coronary intervention can also result in neointimal formation which induces restenosis. Sirolimus-loaded microbubbles were shown to reduce neointimal formation in coronary arteries by 50% in pigs, see Figure 7, 28 days after angioplasty in combination with a mechanically rotating intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative pressure) (Kilroy, et al. 2015). Another research group showed that paclitaxel-loaded microbubbles and ultrasound (1 MHz, 1.5 MPa for 10 s) can also significantly inhibit neointimal formation in the iliac artery in rabbits one week after percutaneous coronary intervention (Zhu, et al. 2016).

In diabetic cardiomyopathy, microbubble-mediated delivery of fibroblast growth factor has shown therapeutic effects. Zhao et al (2016) could prevent diabetic cardiomyopathy in rats by treating the heart with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) and microbubbles co-administered with acidic fibroblast growth factor nanoparticles twice weekly for 12 consecutive weeks. In already established diabetic cardiomyopathy in rats, the same investigators co-administered basic fibroblast growth factor-containing nanoparticles with microbubbles with the same ultrasound treatment, albeit that it was given three times with one day in between treatments. At four weeks after treatment, this resulted in restored cardiac

functions as a result of structural remodeling of the cardiac tissue (Zhao, et al. 2014). Microbubbles loaded with acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) also showed significantly improved cardiac function in a rat model of diabetic cardiomyopathy. Treatment was performed twice weekly for 12 consecutive weeks (Zhang, et al. 2016a). For doxorubicin induced cardiomyopathy, repeated co-administration of microbubbles and nanoparticles containing acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) applied at the heart successfully prevented doxorubicin induced cardiomyopathy in rats (Tian, et al. 2017). Once doxorubicin induced cardiomyopathy had occurred, microbubble-mediated reversal of cardiomyopathy was shown by the delivery of survivin plasmid to cardiomyocytes and endothelial cells (Lee, et al. 2014) or glucagon-like peptide-1 (GLP-1) to cardiomyocytes, endothelial cells, vascular muscle cells, and mesenchymal cells (Chen, et al. 2015) in rats. The ultrasound settings were 5 MHz (120 V power, pulsing interval of 10 cardiac cycles at end-systole) for a 5 min treatment (Lee, et al. 2014) or not specified (Chen, et al. 2015). The microbubble-mediated gene therapy study by Chen et al. (2016) showed that ANGPTL8 gene therapy does not need to be done in the heart to reverse doxorubicin induced cardiomyopathy in rats as their microbubble and ultrasound (1.3 MHz, 1.4 MPa peak negative pressure, four bursts triggered to every fourth end-systole using a delay of 45-70 ms of the peak of the R wave) therapy was done in the liver (90 s treatment). This resulted in overexpression of ANGPTL8 in liver cells and blood which stimulated cardiac progenitor cells in the epicardium.

A few dozen articles have been published on treating myocardial infarction with microbubble and ultrasound-mediated gene delivery *in vivo*, in mouse, rat, rabbit, and dog models. These are reviewed by Qian et al. (2018). Amongst these are a few targeted microbubble studies which all show that the targeted microbubbles induced higher degrees of

gene transfection, increased myocardial vascular density, and improved cardiac function in comparison to non-targeted microbubbles. This improvement occurred independent of the type of ligand on the microbubble, the gene that was transfected, or the animal model: matrix metalloproteinase 2 target with Timp3 gene in rats (Yan, et al. 2014), intracellular adhesion molecule-1 target with Ang-1 gene in rabbits (Deng, et al. 2015), P-selectin target with hVEGF165 gene in rats (Shentu, et al. 2018). Ultrasound settings for these studies were similar at 1.6 MHz (1.6 MPa peak negative pressure, pulsing interval of four cardiac cycles) for 20 min during infusion of the plasmid-loaded microbubbles (both Yan et al. (2014) and Shentu et al. (2018)), or 1.7 MHz (1.7 MPa peak negative pressure, pulsing interval every four to eight cardiac cycles) for 5 min after bolus injection of the plasmid-loaded microbubbles (Deng, et al. 2015).

Other gene therapy studies for vascular disease include stimulating angiogenesis for the treatment of chronic hindlimb ischemia in rats using miR-126-3p-loaded microbubbles and ultrasound (1.3 MHz, 2.1 MPa peak negative acoustic pressure, pulsing interval 5 s). The treatment lasted for 20 min of which microbubbles were infused for 10 min and resulted in improved perfusion, vessel density, arteriolar formation, and neovessel maturation (Cao, et al. 2015). Recently, successful gene therapy was demonstrated in baboons where Vascular Endothelial Growth Factor (VEGF)-plasmid loaded microbubbles were infused and ultrasound (2-6 MHz, MI 1.9, repeated 5 s burst pulses with three bursts per minute) was applied for 10 min on days 25, 35, 45, and 55 of gestation with the transducer placed over the placental basal plate (Babischkin, et al. 2019). This was a mechanistic study elucidating the role of VEGF in uterine artery remodeling.

The gas core of the cavitation nuclei can also be the therapeutic. Sutton et al. (2014) have shown that ultrasound-mediated (1 MHz, 0.34 MPa acoustic pressure, 30 cycle pulse, 50 s treatment) nitric oxide gas delivery from echogenic liposomes to *ex vivo* perfused porcine

carotid arteries induces potent vasorelaxation. The vasodialative effect of nitric oxide-loaded echogenic liposomes upon insonification (5.7 MHz, 0.36 MPa peak negative pressure, 30 s treatment) was also shown in *ex vivo* perfused rabbit carotid arteries with arterial wall penetration of nitric oxide confirmed by fluorescence microscopy (Kim, et al. 2014). In addition to this, vasodialative effects were demonstrated in carotid arteries *in vivo* in rats with vasospasms following subarachnoid hemorrhage using 1 MHz ultrasound with 0.3 MPa peak-to-peak pressure, 50% duty cycle for a duration of 40 min with constant infusion of the echogenic liposomes. This resulted in improved neurological function (limb placement, beam and grid walking) (Kim, et al. 2014). Ultrasound-activation of the antioxidant hydrogen gas encapsulated in microbubbles was shown to prevent myocardial ischemia-reperfusion injury in rats when administered before reperfusion (He, et al. 2017). There was a dose-dependent effect as 2×10^{10} microbubbles resulted in a more significant reduction in infarct size (70%) than 4×10^{9} microbubbles (39%) compared to vehicle-treated rats. Furthermore, treatment with the high dose hydrogen-microbubbles prevented changes in left ventricular end-diastolic and left ventricular end-systolic dimension as well as minimal reductions in ejection fraction and fractional shortening. Histological and ELISA analysis showed a reduced degree of myocardial necrosis, apoptosis, hemorrhaging, inflammation, and oxidant damage. At the same time that cardiovascular drug delivery and therapy using microbubbles and ultrasound is moving forward to large animal and clinical studies, sophisticated *in vitro* models are being used and/or developed for mechanistic studies, such as flow chambers (µSlides, Ibidi) (Shamout, et al. 2015) and perfused 3D microvascular networks (Juang, et al. 2019) in which human umbilical vein endothelial cells are grown.

Clinical study

Microbubbles and ultrasound were clinically investigated to augment muscle blood flow in 12 patients with stable sickle cell disease in the absence of a drug at the Oregon Health & Science University, Portland, Oregon, USA (Belcik, et al. 2017). Perfusion increased ~2-fold in the forearm flexor muscles upon Definity® infusion and insonification at 1.3 MHz (MI 1.3). Ultrasound was applied 3 times for 3 min with ~5 min intervals. The change in perfusion was determined from contrast enhanced ultrasound imaging and extended well beyond the region where ultrasound was applied. This study showed that the therapeutic ultrasound settings directly translate from mouse to man for superficial muscles, as the same investigators demonstrated augmented blood flow in ischemic and non-ischemic hindlimb muscles in mice in the same study and an earlier publication (Belcik, et al. 2015). However, for the preclinical studies custom-made microbubbles were used instead of Definity®.

SONOBACTERICIDE

Sonobactericide has been defined as the use of ultrasound in the presence of cavitation nuclei for the enhancement of bactericidal action (Lattwein, et al. 2018). This topic has recently gained attention with 17 papers being published in the last five years. Research on ultrasound-mediated enhancement of antimicrobials has focused on several sources of infections including general medical devices (Ronan, et al. 2016, Dong, et al. 2017, Dong, et al. 2018, Hu, et al. 2018, Fu, et al. 2019), acne (Liao, et al. 2017), chronic bacterial prostatitis (Yi, et al. 2016), infective endocarditis (Lattwein, et al. 2018), pneumonia (Sugiyama, et al. 2018), prosthetic joint infections (Li, et al. 2015, Lin, et al. 2015, Guo, et al. 2017a, Zhou, et al. 2018), or urinary tract infections (Horsley, et al. 2019). However, there was no specific disease aim in two studies (Zhu, et al. 2014, Goh, et al. 2015). One group targeted membrane biofouling for water and wastewater industries (Agarwal, et al. 2014). Direct bacterial killing, biofilm degradation and dispersal, and increased or synergistic therapeutic effectiveness of antimicrobials have been

reported as the therapeutic effects of sonobactericide. These studies show that sonobactericide can be applied to treat Gram+ or Gram– bacteria, when they are planktonic, associated with a surface and embedded in biofilm, or intracellular. The majority of these studies were carried out *in vitro*. However, seven were performed *in vivo* in either mice (Li, et al. 2015, Liao, et al. 2017, Sugiyama, et al. 2018, Zhou, et al. 2018), rats (Yi, et al. 2016), or rabbits (Lin, et al. 2015, Dong, et al. 2018). Sonobactericide was mostly performed with co-administration of antimicrobials. Investigators also employed an antimicrobial encapsulated in liposomes that were conjugated to the microbubbles (Horsley, et al. 2019), or the antimicrobial lysozyme was a microbubble coating (Liao, et al. 2017), or did not use antimicrobials altogether (Agarwal, et al. 2014, Goh, et al. 2015, Yi, et al. 2016). An extensive review of sonobactericide has been published recently by Lattwein et al. (2019). Although sonobactericide is an emerging strategy to treat bacterial infections with intriguing potential, the mechanism and the safety of the treatment should be explored, particularly regarding biofilm degradation and dispersal. Future studies should also focus on maximizing the efficacy of sonobactericide *in situ*.

FUTURE PERSPECTIVES AND CONCLUSIONS

Therapeutic ultrasound technology is experiencing a paradigm shift in terms of both technical developments and clinical applications. In addition to its inherent advantages for imaging (e.g., real time nature, portability and low cost), ultrasound in combination with cavitation nuclei is under exploration as a drug delivery modality. The results from several preclinical studies have already demonstrated the potential of ultrasound-responsive cavitation nuclei to deliver multiple types of drugs (including model drugs, anticancer, therapeutic antibodies, genes, nanoparticles, etc.) efficiently in various tumor models, including both ectopic and orthotopic models, for immunotherapy, brain disease, to promote the dissolution of clots, and in the treatment of cardiovascular disease and bacterial infections.

Based on these encouraging preclinical data, several clinical trials have been initiated and others are planned. However, whilst animal studies provide proof of concept, and impetus for clinical studies, careful attention must be given to their relevance in human disease; in particular, the applicability of therapeutic protocols, and appropriate ultrasound settings. Otherwise we risk underestimating the therapeutic effects and potential deleterious side effects. The elucidation of all of the interactions between cavitation nuclei – cells and drugs will help to address this need. The biggest challenges lie in the large differences in timescales between the cavitation nuclei, drug release and uptake, and the biological response (Figure 8). A multidisciplinary approach is needed to tackle these challenges integrating expertise in physics, biophysics, biology, chemistry, and pharmacology.

Custom-made microbubbles which serve as cavitation nuclei are often used for ultrasound-mediated drug delivery studies. An advantage is full control over the payload, as well as the disease target. At the same time, full acoustical characterization and sterility of the microbubbles must be considered during translation to human studies, which often requires approval from the United States Food and Drug Administration (FDA) or other similar federal agencies in Europe and Asia. As an example, for gene therapy, will each different type of genetic material loaded onto microbubbles need such approval, or will a class of cationic microbubbles be approved regardless of the specific gene? The former path would hinder fast clinical translation. For now, co-administration of drugs with FDA-approved ultrasound contrast agents is being explored in clinical trials. Apart from applications in the brain, ongoing clinical studies evaluating microbubble-mediated drug delivery are based on standard clinical ultrasound scanners operating mostly in Doppler mode. In order to promote the progress of this emerging technology, it is very important to design and implement specific therapeutic ultrasound pulse sequences that might be vastly different from clinical diagnostic imaging output. Clinical scanners can indeed be modified to be able to generate drug delivery protocols.

In a similar way that elastography requires long ultrasound pulses to generate the push sequences (Deffieux, et al. 2009) , ultrasound scanners can be modified to be able to transmit drug delivery ultrasound sequences with tailored and optimized parameters (pulse duration, duty cycle, and center frequency).

Ultimately, ultrasound image-guided drug delivery and the monitoring of treatment response could be feasible with the same equipment. Additionally, with recent developments in ultrasound imaging technology, ultrasound-mediated therapy could be planned, applied and monitored in a rapid sequence with high spatial and temporal resolution. The use of a single imaging and therapy device would alleviate the need for co-registration, because the imaging equipment would also be used to induce localized therapy ensuring a perfect co-location. Nonetheless, a compromise between efficacy and safety remains a major challenge for successful clinical applications of this dual methodology, which combines real-time image guidance of therapeutic delivery.

In conclusion, ultrasound-responsive microbubbles which serve as cavitation nuclei are being used to treat a wide variety of diseases and show great potential preclinically and clinically. The elucidation of the cavitation nuclei – cell – interaction and the implementation of drug delivery ultrasound sequences on clinical ultrasound scanners are expected to invigorate clinical studies.

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FIGURE CAPTIONS LIST

Figure 1. Combined effect of nonlinear propagation and focusing of the harmonics in a perfluoropentane micrometer-sized droplet. The emitted ultrasound wave has a frequency of 3.5 MHz and a focus at 3.81 cm, and the radius of the droplet is 10 μm for ease of observation. The pressures are given on the axis of the droplet along the propagating direction of the ultrasound wave, and the shaded area indicates the location of the droplet (reprinted with permission from Sphak et al. (2014)).

Figure 2. Ultrasound-activated microbubbles can locally alter the tumor microenvironment through four mechanisms: enhanced permeability, improved contact, reduced hypoxia, and altered perfusion.

Figure 3. Schematic overview of how microbubbles and ultrasound have been shown to contribute to cancer immunotherapy. From left to right: microbubbles can be used as antigen carriers to stimulate antigen uptake by dendritic cells. Microbubbles and ultrasound can alter the permeability of tumors thereby increasing the intratumoral penetration of adoptively transferred immune cells or checkpoint inhibitors. Finally, exposing tissues to cavitating microbubbles can induce sterile inflammation by the local release of DAMPS.

Figure 4. 3D transcranial subharmonic microbubble imaging and treatment control *in vivo* in rabbit brain during BBB opening. Spectral information (top) shows the appearance of subharmonic activity at t = 35 s into the treatment. Passive mapping of the subharmonic band localizes this activity to the target region. Scale bar indicates 2.5 mm (reprinted (adapted) with permission from Jones et al. (2018)).

Figure 5. T_1 weighted sagittal MR images showing leptomeningeal tumors in rat spinal cord (grey arrowheads) before ultrasound and microbubble treatment (left column), and the enhancement of the cord indicating BSCB opening (white arrows) post-ultrasound and microbubble treatment (right column) (reprinted (adapted) with permission from O'Reilly et al. (2018)).

Figure 6. Simulated acoustic pressure and temperature in a representative subject exposed to pulsed 220 kHz ultrasound with a 33.3% duty cycle. The absolute peak-to-peak pressure maximum for the simulations is displayed in gray scale. Temperature is displayed using a heat map with a minimum color priority write threshold of 1 °C. Computed tomography features such as bone (cyan), skin and internal epithelium (beige), and clot (green), are plotted using contour lines. The transducer is outlined in magenta. Constructive interference is prominent in the soft tissue between the temporal bone and the transducer. Some constructive interference is also present in the brain tissue close to the contralateral temporal bone, however, the pressure in this region did not exceed the pressure in the M1 section of the middle cerebral artery. Temperature rise was prominent in the ipsilateral bone along the transducer axis. Computational model is described in Kleven et al. (2019).

Figure 7. Histological sections of a coronary artery of a pig 28 days after angioplasty. Pigs were treated with sirolimus-loaded microbubbles only (a) or sirolimus-loaded microbubbles and ultrasound (b) using a mechanically rotating intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative pressure). Treatment with ultrasound and sirolimus-loaded microbubbles reduced neointimal formation by 50%. In both sections the intima (I) and media (M) are outlined; scale bar is 500 μm (Reprinted by permission from Springer Nature: Springer, Annals of Biomedical Engineering, Reducing Neointima Formation in a Swine Model with IVUS and Sirolimus Microbubbles, Kilroy JP, Dhanaliwala AH, Klibanov AL, Bowles DK, Wamhoff BR, Hossack JA, COPYRIGHT (2015)).

Figure 8. Different time scales of the therapeutic effects of ultrasound and cavitation nuclei treatment. $[Ca^{2+}]_i$ = intracellular calcium; ROS = reactive oxygen species; ATP = adenosine

triphosphate; EV = extracellular vesicles (reprinted (adapted) with permission from Lattwein et al. (2019)).

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Ultrasound-responsive cavitation nuclei for therapy and drug delivery

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ABSTRACT

Therapeutic ultrasound strategies are actively under development to harness the mechanical activity of cavitation nuclei for beneficial tissue bioeffects. The mechanical oscillations of circulating microbubbles, the most widely investigated cavitation nuclei, which may also encapsulate or shield a therapeutic agent in the bloodstream, trigger and promote localized uptake. Oscillating microbubbles can create stresses either on nearby tissue or in surrounding fluid to enhance drug penetration and efficacy in the brain, spinal cord, vasculature, immune system, biofilm, or tumors. This review summarizes recent investigations that have elucidated interactions of ultrasound and cavitation nuclei with cells, the treatment of tumors, immunotherapy, the blood brain barrier and blood spinal cord barrier, sonothrombolysis, cardiovascular drug delivery, and sonobactericide. In particular, an overview of salient ultrasound features, drug delivery vehicles, therapeutic transport routes, and preclinical and clinical studies is provided. Successful implementation of ultrasound and cavitation nuclei-mediated drug delivery has the potential to change the way drugs are administered systemically, resulting in more effective therapeutics and less-invasive treatments.

Key words: Ultrasound, Cavitation nuclei, Therapy, Drug delivery, Bubble-cell interaction, Sonoporation, Sonothrombolysis, Blood-brain barrier opening, Sonobactericide, Tumor.

INTRODUCTION

Around the start of the European Symposium on Ultrasound Contrast Agents (ESUCI), ultrasound-responsive cavitation nuclei were reported to have therapeutic potential. Thrombolysis was shown to be accelerated *in vitro* (Tachibana and Tachibana 1995) and cultured cells were transfected with plasmid DNA (Bao, et al. 1997). Since then, many research groups have investigated the use of cavitation nuclei for multiple forms of therapy, including both tissue ablation and drug and gene delivery. In the early years, the most widely investigated cavitation nuclei were gas microbubbles, ~1-10 µm in diameter and coated with a stabilizing shell, whereas nowadays both solid and liquid nuclei are also investigated that can be as small as a few hundred nm. Drugs can be co-administered with the cavitation nuclei or loaded in or on them (Lentacker, et al. 2009, Kooiman, et al. 2014). The diseases that can be treated with ultrasound-responsive cavitation nuclei include but are not limited to cardiovascular disease and cancer (Sutton, et al. 2013, Paefgen, et al. 2015), the current leading causes of death worldwide according to the World Health Organization (Nowbar, et al. 2019). This review focuses on the latest insights into cavitation nuclei for therapy and drug delivery from the physical and biological mechanisms of bubble-cell interaction to preclinical (both *in vitro* and *in vivo*) and clinical studies (timespan 2014-2019), with particular emphasis on the key clinical applications. The applications covered in this review are the treatment of tumors, immunotherapy, the blood brain barrier and blood spinal cord barrier, dissolution of clots, cardiovascular drug delivery, and the treatment of bacterial infections.

CAVITATION NUCLEI FOR THERAPY

The most widely used cavitation nuclei are phospholipid-coated microbubbles with a gas core. For the 128 preclinical studies included in the treatment sections of this review, the commercially available and clinically approved Definity® (Luminity® in Europe;

79 octafluoropropane gas core, phospholipid coating) (Definity® 2011, Nolsøe and Lorentzen
80 2016) microbubbles were used the most (in 22 studies). Definity® was used for studies on all
81 applications discussed here and the most for opening the blood brain barrier (BBB) (12
82 studies). SonoVue™ (Lumason® in the USA) is commercially available and clinically
83 approved as well (sulfur hexafluoride gas core, phospholipid coating) (Lumason® 2016, Nolsøe
84 and Lorentzen 2016) and was used in a total of 14 studies for the treatment of non-brain tumors
85 (for example Xing et al. (2016)), BBB opening (for example Goutal et al. (2018)), and
86 sonobactericide (for example Hu et al. (2018)). Other commercially available microbubbles
87 were used that are not clinically approved, such as BR38 (Schneider, et al. 2011) in the study
88 by Wang et al. (2015d) and MicroMarker (VisualSonics) in the study by Theek et al. (2016).
89 Custom-made microbubbles are as diverse as their applications, with special characteristics
90 tailored to enhance different therapeutic strategies. Different types of gasses were used as the
91 core such as air (for example Eggen et al. (2014)), nitrogen (for example Dixon et al. (2019)),
92 oxygen (for example Fix et al. (2018)), octafluoropropane (for example Pandit et al. (2019)),
93 perfluorobutane (for example Dewitte et al. (2015)), sulfur hexafluoride (Bae, et al. 2016,
94 Horsley, et al. 2019) or a mixture of gases such as nitric oxide and octafluoropropane (Sutton,
95 et al. 2014) or sulfur hexafluoride and oxygen (McEwan, et al. 2015). While fluorinated gases
96 improve the stability of phospholipid-coated microbubbles (Rossi, et al. 2011), other gases can
97 be loaded for therapeutic applications, such as oxygen to treat tumors (McEwan, et al. 2015,
98 Fix, et al. 2018, Nesbitt, et al. 2018) and nitric oxide (Kim, et al. 2014, Sutton, et al. 2014) or
99 hydrogen gas (He, et al. 2017) for treatment of cardiovascular disease. The main phospholipid
100 component of custom-made microbubbles is usually a phosphatidylcholine such as 1,2-
101 dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), used in 13 studies, for example Dewitte et
102 al. (2015), Bae et al. (2016), Chen et al. (2016), Fu et al. (2019), or 1,2-distearoyl-*sn*-glycero-
103 3-phosphocholine (DSPC), used in 18 studies, for example Kilroy et al. (2014), Bioley et al.

(2015), Dong et al. (2017), Goyal et al (2017), Pandit et al. (2019). These phospholipids are popular because they are also the main component in Definity® (Definity® 2011) and SonoVue®/Lumason® (Lumason® 2016), respectively. Another key component of the microbubble coating is a PEGylated emulsifier such as polyoxyethylene (40) stearate (PEG40-stearate; for example Kilroy et al. (2014)) or the most often used 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy (poly-ethyleneglycol) (DSPE-PEG2000; for example Belcik et al. (2017)), which is added to inhibit coalescence and to increase the *in vivo* half-life (Ferrara, et al. 2009). In general two methods are used to produce custom-made microbubbles: mechanical agitation (for example Ho et al. (2018)) or probe sonication (for example Belcik et al. (2015)). Both these methods produce a population of microbubbles that is polydisperse in size. Monodispersed microbubbles produced by microfluidics have recently been developed, and are starting to gain attention for pre-clinical therapeutic studies. Dixon et al. (2019) used monodisperse microbubbles to treat ischemic stroke.

Various therapeutic applications have inspired the development of novel cavitation nuclei, which is discussed in depth in the companion review by Stride et al. (2019). To improve drug delivery, therapeutics can be either co-administered with or loaded onto the microbubbles. One strategy for loading is to create microbubbles stabilized by drug-containing polymeric nanoparticles around a gas core (Snipstad, et al. 2017). Another strategy is to attach therapeutic molecules or liposomes to the outside of microbubbles, for example by biotin-avidin coupling (Dewitte, et al. 2015, McEwan, et al. 2016, Nesbitt, et al. 2018). Echogenic liposomes can be loaded with different therapeutics or gases and have been studied for vascular drug delivery (Sutton, et al. 2014), treatment of tumors (Choi, et al. 2014), and sonothrombolysis (Shekhar, et al. 2017). ACT® combines Sonazoid® microbubbles with droplets that can be loaded with therapeutics for treatment of tumors (Kotopoulis, et al. 2017). The cationic microbubbles utilized in the treatment sections of this review were used mostly for vascular drug delivery,

with genetic material loaded on the microbubble surface by charge-coupling (for example Cao et al. (2015)). Besides phospholipids and nanoparticles, microbubbles can also be coated with denatured proteins such as albumin. Optison™ (Optison™ 2012) is a commercially available and clinically approved ultrasound contrast agent that is coated with human albumin and used in studies on treatment of non-brain tumors (Xiao, et al. 2019), BBB opening (Kovacs, et al. 2017b, Payne, et al. 2017), and immunotherapy (Maria, et al. 2015). Nano-sized particles cited in this review have been used as cavitation nuclei for treatment of tumors, such as nanodroplets (for example Cao et al. (2018)) and nanocups (Myers, et al. 2016), for BBB opening (nanodroplets, Wu et al. (2018)), and for sonobactericide (nanodroplets, Guo et al. (2017a)).

BUBBLE-CELL INTERACTION

Physics

The physics of the interaction between bubbles or droplets and cells are described as these are the main cavitation nuclei used for drug delivery and therapy.

Physics of Microbubble – Cell Interaction

Being filled with gas and/or vapor makes bubbles highly responsive to changes in pressure and hence exposure to ultrasound can cause rapid and dramatic changes in their volume. These volume changes in turn give rise to an array of mechanical, thermal, and chemical phenomena that can significantly influence the bubbles' immediate environment and mediate therapeutic effects. For the sake of simplicity, these phenomena will be discussed in the context of a single bubble. It is important to note, however, that biological effects are typically produced by a population of bubbles and the influence of inter bubble interactions should not be neglected.

a. Mechanical effects

A bubble in a liquid is subject to multiple competing influences: the driving pressure of the imposed ultrasound field, the hydrostatic pressure imposed by the surrounding liquid, the

pressure of the gas and/or vapor inside the bubble, surface tension and the influence of any coating material, the inertia of the surrounding fluid, and damping due to the viscosity of the surrounding fluid and/or coating, thermal conduction, and/or acoustic radiation.

The motion of the bubble is primarily determined by the competition between the liquid inertia and the internal gas pressure. This competition can be characterized by using the Rayleigh-Plesset equation for bubble dynamics to compare the relative contributions of the terms describing inertia and pressure to the acceleration of the bubble wall (Flynn 1975a):

$$\ddot{R} = -\left(\frac{3}{2}\frac{\dot{R}^2}{R}\right) + \left(\frac{p_G(R)+p_\infty(t)-\frac{2\sigma}{R}}{\rho_L R}\right) = IF + PF, \quad \text{(Eq. 1)}$$

where R is the time dependent bubble radius with initial value R_o, p_G is the pressure of the gas inside the bubble, p_∞ is the combined hydrostatic and time varying pressure in the liquid, σ is the surface tension at the gas liquid interface, and ρ_L is the liquid density.

Flynn (1975b, a) identified two scenarios: if the pressure factor (PF) is dominant when the bubble approaches its minimum size, then the bubble will undergo sustained volume oscillations. If the inertia term is dominant (IF), then the bubble will undergo inertial collapse, similar to an empty cavity, after which it may rebound or it may disintegrate. Which of these scenarios occurs is dependent upon the bubble expansion ratio: R_{max}/R_o, and hence the bubble size and the amplitude and frequency of the applied ultrasound field.

Both inertial and non-inertial bubble oscillations can give rise to multiple phenomena that impact the bubble's immediate environment and hence are important for therapy. These include:

(i) Direct impingement – even at moderate amplitudes of oscillation, the acceleration of the bubble wall may be sufficient to impose significant forces upon nearby surfaces, easily

deforming fragile structures such as a biological cell membranes (van Wamel, et al. 2006, Kudo 2017) or blood vessel walls (Chen, et al. 2011).

(ii) Ballistic motion – in addition to oscillating, the bubble may undergo translation as a result of the pressure gradient in the fluid generated by a propagating ultrasound wave (primary radiation force). Due to their high compressibility, bubbles may travel at significant velocities, sufficient to push them toward targets for improved local deposition of a drug (Dayton, et al. 1999) or penetrate biological tissue (Caskey, et al. 2009, Bader, et al. 2015, Acconcia, et al. 2016).

(iii) Microstreaming – when a structure oscillates in a viscous fluid there will be a transfer of momentum due to interfacial friction. Any asymmetry in the oscillation will result in a net motion of that fluid in the immediate vicinity of the structure known as microstreaming (Kolb and Nyborg 1956). This motion will in turn impose shear stresses upon any nearby surfaces as well as increasing convection within the fluid. Due to the inherently non-linear nature of bubble oscillations (equation 1), both non-inertial and inertial cavitation can produce significant microstreaming, resulting in fluid velocities on the order of 1 mm/s (Pereno and Stride 2018). If the bubble is close to a surface then it will also exhibit non-spherical oscillations which increases the asymmetry and hence the microstreaming even further (Nyborg 1958, Marmottant and Hilgenfeldt 2003).

(iv) Microjetting – another phenomenon associated with non-spherical bubble oscillations near a surface is the generation of a liquid jet during bubble collapse. If there is sufficient asymmetry in the acceleration of the fluid on either side of the collapsing bubble, then the more rapidly moving fluid may deform the bubble into a toroidal shape causing a high velocity jet to be emitted on the opposite side. Microjetting has been shown to be capable of producing pitting even in highly resilient materials such as steel (Naudé and Ellis 1961, Benjamin and Ellis 1966). However, as both the direction and velocity of the jet are determined by the elastic

properties of the nearby surface, its effects in biological tissue are more difficult to predict (Kudo and Kinoshita 2014). Nevertheless, as shown by Chen et al. (2011), in many cases a bubble will be sufficiently confined that microjetting will impact surrounding structures regardless of jet direction.

(v) Shockwaves – an inertially collapsing cavity that results in supersonic bubble wall velocities creates a significant discontinuity in the pressure in the surrounding liquid leading to the emission of a shockwave, which may impose significant stresses on nearby structures.

(vi) Secondary radiation force – at smaller amplitudes of oscillation a bubble will also generate a pressure wave in the surrounding fluid. If the bubble is adjacent to a surface, interaction between this wave and its reflection from the surface leads to a pressure gradient in the liquid and a secondary radiation force on the bubble. As with microjetting, the elastic properties of the boundary will determine the phase difference between the radiated and reflected waves and hence whether the bubbles move towards or away from the surface. Motion towards the surface may amplify the effects of (i), (iii), and (vi).

b. Thermal effects

As described above, an oscillating microbubble will reradiate energy from the incident ultrasound field in the form of a spherical pressure wave. In addition, the nonlinear character of the microbubble oscillations will lead to energy being reradiated over a range of frequencies. At moderate driving pressures the bubble spectrum will contain integer multiples (harmonics) of the driving frequency; and at higher pressures also fractional components (sub and ultraharmonics). In biological tissue, absorption of ultrasound increases with frequency and this nonlinear behavior thus also increases the rate of heating (Hilgenfeldt, et al. 2000, Holt and Roy 2001). Bubbles will also dissipate energy as a result of viscous friction in the liquid and thermal conduction from the gas core, the temperature of which increases during compression. Which mechanism is dominant depends on the size of the bubble, the driving

conditions and the viscosity of the medium. Thermal damping is however typically negligible in biomedical applications of ultrasound as the time constant associated with heat transfer is much longer than the period of the microbubble oscillations (Prosperetti 1977).

c. Chemical effects

The temperature rise produced in the surrounding tissue will be negligible compared with that occurring inside the bubble, especially during inertial collapse when it may reach several thousand Kelvin (Flint and Suslick 1991). The gas pressure similarly increases significantly. While only sustained for a very brief period, these extreme conditions can produce highly reactive chemical species, in particular reactive oxygen species (ROS), as well as the emission of electromagnetic radiation (sonoluminescence). ROS have been shown to play a significant role in multiple biological processes (Winterbourn 2008) and both ROS and sonoluminescence may affect drug activity (Rosenthal, et al. 2004, Trachootham, et al. 2009, Beguin, et al. 2019).

Physics of Droplets – Cell Interaction

Droplets consist of an encapsulated quantity of a volatile liquid, such as perfluorobutane (boiling point -1.7 °C) or perfluoropentane (boiling point 29 °C), which is in a superheated state at body temperature. Superheated state means that although the volatile liquids have a boiling point below 37 °C, these droplets remain in the liquid phase and do not show spontaneous vaporization after injection. Vaporization can be achieved instead by exposure to ultrasound of significant amplitude via a process known as acoustic droplet vaporization (ADV) (Kripfgans, et al. 2000). Before vaporization, the droplets are typically one order of magnitude smaller than the emerging bubbles, and the perfluorocarbon is inert and biocompatible (Biro and Blais 1987). These properties enable a range of therapeutic possibilities (Sheeran and Dayton 2012, Lea-Banks, et al. 2019). For example, unlike microbubbles, small droplets may extravasate from the leaky vessels into tumor tissue due to

the enhanced permeability and retention (EPR) effect (Long, et al. 1978, Lammers, et al. 2012, Maeda 2012), and then be turned into bubbles by ADV (Rapoport, et al. 2009, Kopechek, et al. 2013). Loading the droplets with a drug enables local delivery (Rapoport, et al. 2009) by way of ADV. The mechanism behind this is that the emerging bubbles give rise to similar radiation forces and microstreaming as described in the physics of the microbubble – cell interaction above. It should be noted that oxygen is taken up during bubble growth (Radhakrishnan, et al. 2016), which could lead to hypoxia.

The physics of the droplet – cell interaction is largely governed by the ADV. In general, it has been observed that ADV is promoted by the following factors: large peak negative pressures (Kripfgans, et al. 2000), usually obtained by strong focusing of the generated beam, high frequency of the emitted wave, and a relatively long distance between the transducer and the droplet. Another observation that has been made with micrometer-sized droplets is that vaporization often starts at a well-defined nucleation spot near the side of the droplet where the acoustic wave impinges (Shpak, et al. 2014). These facts can be explained by considering the two mechanisms that play a role in achieving a large peak negative pressure inside the droplet: acoustic focusing and nonlinear ultrasound propagation (Shpak, et al. 2016). In the following, lengths and sizes are related to the wavelength, i.e. the distance traveled by a wave in one oscillation (e.g., a 1 MHz ultrasound wave that is traveling in water with a wave speed, *c*, of 1500 m/s has a wavelength, *w* (m), of $\frac{c}{f} = \frac{1500}{10^6} = 0.0015$, *i.e.* 1.5 mm).

a. Acoustic focusing

Because the speed of sound in perfluorocarbon liquids is significantly lower than in water or tissue, refraction of the incident wave will occur at the interface between these fluids, and the spherical shape of the droplet will give rise to focusing. The assessment of this focusing effect is not straightforward because the traditional way of describing these phenomena with rays that propagate along straight lines (the ray approach) only holds for objects that are much

larger than the applied wavelength. In the current case, the frequency of a typical ultrasound wave used for insonification is in the order of 1-5 MHz, yielding wavelengths in the order of 1500 – 300 µm, while a droplet will be smaller by 2-4 orders of magnitude. Beside this, using the ray approach, the lower speed of sound in perfluorocarbon would yield a focal spot near the backside of the droplet, which is in contradiction to observations. The correct way to treat the focusing effect is to solve the full diffraction problem by decomposing the incident wave, the wave reflected by the droplet, and the wave transmitted into the droplet into a series of spherical waves. For each spherical wave, the spherical reflection and transmission coefficients can be derived. Superposition of all the spherical waves yields the pressure inside the droplet. Nevertheless, when this approach is only applied to an incident wave with the frequency that is emitted by the transducer, this will lead neither to the right nucleation spot nor to sufficient negative pressure for vaporization. Nanoscale droplets may be too small to make effective use of the focusing mechanism and ADV is therefore less dependent on the frequency.

b. Nonlinear ultrasound propagation

High pressure amplitudes, high frequencies, and long propagation distances all promote nonlinear propagation of an acoustic wave (Hamilton and Blackstock 2008). In the time domain, nonlinear propagation manifests itself as an increasing deformation of the shape of the ultrasound wave with distance traveled. In the frequency domain, this translates to increasing harmonic content, i.e. frequencies that are multiples of the driving frequency. The total incident acoustic pressure $p(t)$ at the position of a nanodroplet can therefore be written as

$$p(t) = \sum_{n=1}^{\infty} a_n \cos(n\omega t + \phi_n), \qquad \text{(Eq. 2)}$$

where which n is the number of a harmonic, a_n and ϕ_n are the amplitude and phase of this harmonic, and ω is the angular frequency of the emitted wave. The wavelength of a harmonic wave is a fraction of the emitted wavelength.

The above effects are both important in case of ADV and should therefore be combined. This implies that first the amplitudes and phases of the incident nonlinear ultrasound wave at the droplet location should be computed. Next, for each harmonic, the diffraction problem should be solved in terms of spherical harmonics. Adding the diffracted waves inside the droplet with the proper amplitude and phase will then yield the total pressure in the droplet. Figure 1 shows that the combined effects of nonlinear propagation and diffraction can cause a dramatic amplification of the peak negative pressure in the micrometer-sized droplet, sufficient for triggering droplet vaporization (Shpak, et al. 2014). Moreover, the location of the negative pressure peak also agrees with the observed nucleation spot.

After vaporization has started, the growth of the emerging bubble is limited by inertia and heat transfer. In the absence of the heat transfer limitation, the inertia of the fluid that surrounds the bubble limits the rate of bubble growth, which is linearly proportional to time and inversely proportional to the square root of the density of the surrounding fluid. When inertia is neglected, thermal diffusion is the limiting factor in the transport of heat to drive the endothermic vaporization process of perfluorocarbon, causing the radius of the bubble to increase with the square root of time. In reality, both processes occur simultaneously, where the inertia effect is dominant at the early stage and the diffusion effect is dominant at the later stage of bubble growth. The final size that is reached by a bubble depends on the time that a bubble can expand, i.e. on the duration of the negative cycle of the insonifying pressure wave. It is therefore expected that lower insonification frequencies give rise to larger maximum bubble size. Thus, irrespective of their influence on triggering ADV, lower frequencies would lead to more violent inertial cavitation effects and cause more biological damage, as experimentally observed for droplets with a radius in the order of 100 nm (Burgess and Porter 2019).

Biological mechanisms and bioeffects of ultrasound-activated cavitation nuclei

The biological phenomena of sonoporation (*i.e.* membrane pore formation), stimulated endocytosis, and opening of cell-cell contacts and the bioeffects of intracellular calcium transients, reactive oxygen species generation, cell membrane potential change, and cytoskeleton changes have been observed for several years (Sutton, et al. 2013, Kooiman, et al. 2014, Lentacker, et al. 2014, Qin, et al. 2018b). However, other bioeffects induced by ultrasound-activated cavitation nuclei have recently been discovered. These include membrane blebbing as a recovery mechanism for reversible sonoporation (both for ultrasound-activated microbubbles (Leow, et al. 2015) and upon ADV (Qin, et al. 2018a)), extracellular vesicle formation (Yuana, et al. 2017), suppression of efflux transporters P-glycoprotein (Cho, et al. 2016, Aryal, et al. 2017) and BBB (Blood Brain Barrier) transporter genes (McMahon, et al. 2018). At the same time, more insight has been gained in the origin of the bioeffects, largely through the use of live cell microscopy. For sonoporation, real time membrane pore opening and closure dynamics were revealed with pores <30 μm^2 closing within 1 min, while pores >100 μm^2 did not reseal (Hu, et al. 2013) as well as immediate rupture of filamentary actin at the pore location (Chen, et al. 2014) and correlation of intracellular reactive oxygen species levels with the degree of sonoporation (Jia, et al. 2018). Real-time sonoporation and opening of cell-cell contacts in the same endothelial cells has been demonstrated as well for a single example (Helfield, et al. 2016). The applied acoustic pressure was shown to determine uptake of model drugs via sonoporation or endocytosis in another study (De Cock, et al. 2015). Electron microscopy revealed formation of transient membrane disruptions and permanent membrane structures, *i.e.* caveolar endocytic vesicles, upon ultrasound and microbubble-treatment (Zeghimi, et al. 2015). A study by Fekri et al. (2016) revealed that enhanced clathrin-mediated endocytosis and fluid-phase endocytosis occur through distinct signaling mechanisms upon ultrasound and microbubble treatment. The majority of these bioeffects have

been observed in *in vitro* models using largely non-endothelial cells and may therefore not be directly relevant to *in vivo* tissue, where intravascular micron-sized cavitation nuclei will only have contact with endothelial cells and circulating blood cells. On the other hand, the mechanistic studies by Belcik et al. (2015, 2017) and Yu et al. (2017) do show translation from *in vitro* to *in vivo*. In these studies, ultrasound-activated microbubbles were shown to induce a shear-dependent increase in intravascular adenosine triphosphate (ATP) from both endothelial cells and erythrocytes, an increase in intramuscular nitric oxide, and downstream signaling through both nitric oxide and prostaglandins which resulted in augmentation of muscle blood flow. Ultrasound settings were similar, namely 1.3 MHz, MI 1.3 for Belcik et al. (2015, 2017) and 1 MHz, MI 1.5 for Yu et al. (2017), with MI defined as $MI = \frac{P_-}{\sqrt{f}}$ where P_- is the peak negative pressure of the ultrasound wave (in MPa) and *f* the center frequency of the ultrasound wave (in MHz).

Whether or not there is a direct relationship between the type of microbubble oscillation and specific bioeffects remains to be elucidated, although more insight has been gained through ultra-high-speed imaging of the microbubble behavior in conjunction with live cell microscopy. For example, there seems to be a microbubble excursion threshold above which sonoporation occurs (Helfield, et al. 2016). Van Rooij et al. (2016) further showed that displacement of targeted microbubbles enhanced reversible sonoporation and preserved cell viability whilst microbubbles that did not displace were identified as the main contributors to cell death.

All of the aforementioned biological observations, mechanisms, and effects relate to eukaryotic cells. Study of the biological effects of cavitation on for example bacteria is in its infancy, but studies suggest that sonoporation can be achieved in Gram– bacteria, with dextran uptake and gene transfection being reported in *Fusobacterium nucleatum* (Han, et al. 2007). More recent studies have investigated the effect of microbubbles and ultrasound on gene

expression (Li, et al. 2015, Dong, et al. 2017, Zhou, et al. 2018). The findings are conflicting because although they all show a reduction in expression of genes involved in biofilm formation and resistance to antibiotics, an increase in expression of genes involved with dispersion and detachment of biofilms was also found (Dong, et al. 2017). This cavitation-mediated bioeffect needs further investigation.

Modelling Microbubble – cell – drug interaction

Whilst there have been significant efforts to model the dynamics of ultrasound driven microbubbles (Faez, et al. 2013, Dollet, et al. 2019), less attention has been paid to the interactions between microbubbles and cells or their impact upon drug transport. Currently there are no models that describe the interactions between microbubbles, cells, and drug molecules. Several models have been proposed for the microbubble – cell interaction in sonoporation focusing on different aspects: the cell expansion and microbubble jet velocity (Guo, et al. 2017b), the shear stress exerted on the cell membrane (Wu 2002, Doinikov and Bouakaz 2010, Forbes and O'Brien 2012, Yu and Chen 2014, Cowley and McGinty 2019), microstreaming (Yu and Chen 2014), shear stress exerted on the cell membrane in combination with microstreaming (Li, et al. 2014), or other flow phenomena (Yu, et al. 2015, Rowlatt and Lind 2017) generated by an oscillating microbubble. In contrast to the other models, Man et al. (2019) propose that the microbubble-generated shear stress does not induce pore formation, but that this is instead due to microbubble fusion with the membrane and subsequent "pull out" of cell membrane lipid molecules by the oscillating microbubble. Models for pore formation (for example Koshiyama and Wada (2011)) and resealing (Zhang, et al. 2019) in cell membranes have also been developed, but these models neglect the mechanism by which the pore is created. There is just one sonoporation dynamics model, developed by Fan *et al*. (2012), that relates the uptake of the model drug propidium iodide (PI) to the size of the created

membrane pore and the pore resealing time for a single cell in an *in vitro* setting. The model describes the intracellular fluorescence intensity of PI as a function of time, *F(t)*, by:

$$F(t) = \alpha \cdot \pi D C_0 \cdot r_o \cdot \frac{1}{\beta}\left(1 - e^{-\beta t}\right), \quad \text{(Eq. 3)}$$

where α is the coefficient that relates the amount of PI molecules to the fluorescence intensity of PI-DNA and PI-RNA, D is the diffusion coefficient of PI, C_0 is the extracellular PI concentration, r_0 is the initial radius of the pore, β is the pore resealing coefficient, and t is time. The coefficient α is determined by the sensitivity of the fluorescence imaging system, and if unknown the equation can still be used because it is the pore size coefficient, $\alpha \cdot \pi D C_0 \cdot r_0$, that determines the initial slope of the PI uptake pattern and is the scaling factor for the exponential increase. A cell with a large pore will have a steep initial slope of PI uptake and the maximum PI intensity quickly reaches the plateau value. A limitation of this model is that equation 3 is based on two-dimensional free diffusion models, which holds for PI-RNA but not for PI-DNA because this is confined to the nucleus. The model is independent of cell type, as Fan et al. have demonstrated agreement with experimental results in both kidney (Fan, et al. 2012) and endothelial cells (Fan, et al. 2013). Other researchers have also used this model for endothelial cell studies and also classified the distribution of both the pore size and pore resealing coefficients using Principal Component Analysis to determine whether cells were reversibly or irreversibly sonoporated. In the context of blood brain barrier (BBB) opening, Hosseinkhah et al. (2015) have modeled the microbubble-generated shear and circumferential wall stress for 5 μm microvessels upon microbubble oscillation at a fixed mechanical index (MI) of 0.134 for a range of frequencies (0.5, 1, and 1.5 MHz). The wall stresses were dependent upon microbubble size (range investigated 2 – 18 μm in diameter) and ultrasound frequency. Wiedemair et al. (2017) have also modelled the wall shear stress generated by microbubble (2 μm diameter) destruction at 3 MHz for larger microvessels (200 μm diameter). The presence of red blood cells was included in the model and was found to cause confinement

of pressure and shear gradients to the vicinity of the microbubble. Advances in methods for imaging microbubble-cell interactions will facilitate the development of more sophisticated mechanistic models.

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TREATMENT OF TUMORS (NON-BRAIN)

The structure of tumor tissue varies significantly from that of healthy tissue which has important implications for its treatment. To support the continuous expansion of neoplastic cells, the formation of new vessels (i.e. angiogenesis) is needed (Junttila and de Sauvage 2013). As such, a rapidly-developed, poorly-organized vasculature with enlarged vascular openings arises. In between these vessels, large avascular regions exist, which are characterized by a dense extracellular matrix, high interstitial pressure, low pH, and hypoxia. Moreover, a local immunosuppressive environment is formed, preventing possible anti-tumor activity by the immune system.

Notwithstanding the growing knowledge of the pathophysiology of tumors, treatment remains challenging. Chemotherapeutic drugs are typically administered to abolish the rapidly-dividing cancer cells. Yet, their cytotoxic effects are not limited to cancer cells, causing dose-limiting off-target effects. To overcome this hurdle, chemotherapeutics are often encapsulated in nano-sized carriers, i.e. nanoparticles, that are designed to specifically diffuse through the large openings of tumor vasculature, while being excluded from healthy tissue by normal blood vessels (Lammers, et al. 2012, Maeda 2012). Despite being highly promising in pre-clinical studies, drug-containing nanoparticles have shown limited clinical success due to the vast heterogeneity in tumor vasculature (Barenholz 2012, Lammers, et al. 2012, Wang, et al. 2015d). In addition, drug penetration into the deeper layers of the tumor can be constrained due to high interstitial pressure and a dense extracellular matrix in the tumor. Furthermore,

acidic and hypoxic regions limit the efficacy of radiation- and chemotherapy-based treatments due to biochemical effects (Mehta, et al. 2012, McEwan, et al. 2015, Fix, et al. 2018). Ultrasound-triggered microbubbles are able to alter the tumor environment locally, thereby improving drug delivery to tumors. These alterations are schematically represented in Figure 2 and include: improving vascular permeability, modifying the tumor perfusion, reducing local hypoxia, and overcoming the high interstitial pressure.

Several studies have found that ultrasound-driven microbubbles improved delivery of chemotherapeutic agents in tumors, which resulted in increased anti-tumor effects (Wang, et al. 2015d, Snipstad, et al. 2017, Zhang, et al. 2018). Moreover, several gene products could be effectively delivered to tumor cells via ultrasound-driven microbubbles, resulting in a downregulation of tumor-specific pathways and an inhibition in tumor growth (Kopechek, et al. 2015, Zhou, et al. 2015). Theek et al. (2016) furthermore confirmed that nanoparticle accumulation can be achieved in tumors with low EPR effect. Drug transport and distribution through the dense tumor matrix and into regions with elevated interstitial pressure is often the limiting factor in peripheral tumors. As a result, several reports have indicated that drug penetration into the tumor remained limited after sonoporation, which may impede the eradication of the entire tumor tissue (Eggen, et al. 2014, Wang, et al. 2015d, Wei, et al. 2019). Alternatively, microbubble cavitation can affect tumor perfusion, as vasoconstriction and even temporary vascular shut-down have been reported *ex vivo* (Keravnou, et al. 2016) and *in vivo* (Hu, et al. 2012, Goertz 2015, Yemane, et al. 2018). These effects were seen at higher ultrasound intensities (>1.5 MPa) and are believed to result from inertial cavitation leading to violent microbubble collapses. As blood supply is needed to maintain tumor growth, vascular disruption might form a different approach to cease tumor development. Microbubble-induced microvascular damage was able to complement the direct effects of chemotherapeutics and anti-vascular drugs by secondary ischemia-mediated cytotoxicity, which led to tumor growth

inhibition (Wang, et al. 2015a, Ho, et al. 2018, Yang, et al. 2019b). In addition, a synergistic effect between radiation therapy and ultrasound-stimulated microbubble treatment was observed, as radiation therapy also induces secondary cell death by endothelial apoptosis and vascular damage (Lai, et al. 2016, Daecher, et al. 2017). Nevertheless, several adverse effects have been reported due to excessive vascular disruption, including hemorrhage, tissue necrosis, and the formation of thrombi (Goertz 2015, Wang, et al. 2015d, Snipstad, et al. 2017).

Furthermore, oxygen-containing microbubbles can provide a local oxygen supply to hypoxic areas, rendering oxygen-dependent treatments more effective. This is of interest for sonodynamic therapy, which is based on the production of cytotoxic reactive oxygen species (ROS) by a sonosensitizing agent upon activation by ultrasound in the presence of oxygen (McEwan, et al. 2015, McEwan, et al. 2016, Nesbitt, et al. 2018). As ultrasound can be used to stimulate the release of oxygen from oxygen-carrying microbubbles while simultaneously activating a sonosensitizer, this approach has shown to be particularly useful for the treatment of hypoxic tumor types (McEwan, et al. 2015, Nesbitt, et al. 2018). Additionally, low oxygenation promotes resistance to radiotherapy, which can be circumvented by a momentary supply of oxygen. Based on this notion, oxygen-carrying microbubbles were used to improve the outcome of radiotherapy in a rat fibrosarcoma model (Fix, et al. 2018).

Finally, ultrasound-activated microbubbles promote convection and induce acoustic radiation forces. As such, closer contact with the tumor endothelial and an extended contact time can be obtained (Kilroy, et al. 2014). Furthermore, these forces may counteract the elevated interstitial pressure present in tumors (Eggen, et al. 2014, Lea-Banks, et al. 2016, Xiao, et al. 2019).

Apart from their ability to improve the tumor uptake, microbubbles can be used as ultrasound-responsive drug carriers to reduce the off-target effects of chemotherapeutics. By loading the drugs or drug-containing nanoparticles directly in or onto the microbubbles, a

spatial and temporal control of drug release can be obtained, thereby reducing exposure to other parts of the body (Yan, et al. 2013, Snipstad, et al. 2017). Moreover, several studies have shown improved anti-cancer effects from treatment with drug-coupled microbubbles, compared to a co-administration approach (Burke, et al. 2014, Snipstad, et al. 2017). Additionally, tumor neovasculature expresses specific surface receptors that can be targeted by specific ligands. Adding such targeting moieties to the surface of (drug-loaded) microbubbles improves site-targeted delivery and has shown to potentiate this effect further (Bae, et al. 2016, Xing, et al. 2016, Luo, et al. 2017).

Phase-shifting droplets and gas-stabilizing solid agents (*e.g.* nanocups) have the unique ability to benefit from both EPR-mediated accumulation in the 'leaky' parts of the tumor vasculature due to their small sizes, as well as from ultrasound-induced permeabilization of the tissue structure (Zhou 2015, Myers, et al. 2016, Liu, et al. 2018b, Zhang, et al. 2018). Several research groups have reported tumor regression after treatment with acoustically-active droplets (Gupta, et al. 2015, van Wamel, et al. 2016, Cao, et al. 2018, Liu, et al. 2018b) or gas-stabilizing solid particles (Min, et al. 2016, Myers, et al. 2016). A different approach to the use of droplets for tumor treatment, is Acoustic Cluster Therapy (ACT®), which is based on microbubble-droplet clusters that upon ultrasound exposure, undergo a phase shift to create large bubbles that can transiently block capillaries (Sontum, et al. 2015). While the mechanism behind the technique is not yet fully understood, studies have shown improved delivery and efficacy of paclitaxel and Abraxane® in xenograft prostate tumor models (van Wamel, et al. 2016, Kotopoulis, et al. 2017). Another use of droplets for tumor treatment is enhanced high-intensity focused ultrasound (HIFU)-mediated heating of tumors (Kopechek, et al. 2014).

Although microbubble-based drug delivery to solid tumors shows great promise, it also faces important challenges. The ultrasound parameters used in *in vivo* studies highly vary between research groups and no consensus was found on the oscillation regime that is believed

to be responsible for the observed effects (Wang, et al. 2015d, Snipstad, et al. 2017). Moreover, longer ultrasound pulses and increased exposure times are usually applied in comparison to *in vitro* reports (Roovers, et al. 2019c). This could promote additional effects such as microbubble clustering and microbubble translation, which could cause local damage to the surrounding tissue as well (Roovers, et al. 2019a). To elucidate these effects further, fundamental *in vitro* research remains important. Therefore, novel *in vitro* models that more accurately mimic the complexity of the *in vivo* tumor environment are currently being explored. Park et al. (2016) engineered a perfusable vessel-on-a-chip system and reported successful doxorubicin delivery to the endothelial cells lining this microvascular network. While such microfluidic chips could be extremely useful to study the interactions of microbubbles with the endothelial cell barrier, special care to the material of the chambers should be taken to avoid ultrasound reflections and standing waves (Beekers, et al. 2018). Alternatively, 3D tumor spheroids have been used to study the effects of ultrasound and microbubble-assisted drug delivery on penetration and therapeutic effect in a multicellular tumor model (Roovers, et al. 2019b). Apart from expanding the knowledge on microbubble-tissue interactions in detailed parametric studies *in vitro*, it will be crucial to obtain improved control over the microbubble behavior *in vivo*, and link this to the therapeutic effects. To this end, passive cavitation detection (PCD) to monitor microbubble cavitation behavior in real-time is currently under development, and could provide better insights in the future (Choi, et al. 2014, Graham, et al. 2014, Haworth, et al. 2017). Efforts are being committed to constructing custom-built delivery systems, which can be equipped with multiple transducers allowing drug delivery guided by ultrasound imaging and/or PCD (Escoffre, et al. 2013, Choi, et al. 2014, Wang, et al. 2015c, Paris, et al. 2018).

Clinical studies

Pancreatic cancer

553 The safety and therapeutic potential of improved chemotherapeutic drug delivery using
554 microbubbles and ultrasound was first investigated for the treatment of inoperable pancreatic
555 ductal adenocarcinoma at Haukeland University Hospital, Norway (Kotopoulis, et al. 2013,
556 Dimcevski, et al. 2016). In this clinical trial, gemcitabine was administrated by intravenous
557 injection over 30 min. During the last 10 min of chemotherapy, an abdominal echography was
558 performed to locate the position of pancreatic tumor. At the end of chemotherapy, 0.5 mL of
559 SonoVue® microbubbles followed by 5 mL saline were intravenously injected every 3.5 min
560 to ensure their presence throughout the whole sonoporation treatment. Pancreatic tumors were
561 exposed to ultrasound (1.9 MHz, MI 0.2, 1% DC) using a 4C curvilinear probe (GE Healthcare)
562 connected to an LOGIQ 9 clinical ultrasound scanner. The cumulative ultrasound exposure
563 was only 18.9 s. All clinical data showed that microbubble-mediated gemcitabine delivery did
564 not induce any serious adverse events in comparison to chemotherapy alone. At the same time,
565 tumor size and development were characterized according to the Response Evaluation Criteria
566 in Solid Tumors (RECIST) criteria. In addition, Eastern Cooperative Oncology Group (ECOG)
567 performance status was used to monitor the therapeutic efficacy of the microbubble-mediated
568 gemcitabine delivery. All ten patients tolerated an increased number of gemcitabine cycles
569 compared to treatment with chemotherapy alone from historical controls (8.3 ± 6 *vs* 13.8 ± 5.6
570 cycles; $p < 0.008$), thus reflecting an improved physical state. After 12 treatment cycles, one
571 patient's tumor showed a 2-fold decrease in tumor size. This patient was excluded from this
572 clinical trial to be treated with radiotherapy and then with pancreatectomy. In five out of ten
573 patients, the maximum tumor diameter was partially decreased from the first to last therapeutic
574 treatment. Subsequently, a consolidative radiotherapy or a FOLFIRINOX treatment, a bolus
575 and infusion of 5-fluorouracil, leucovorin, irinotecan, and oxaliplatin, was offered to them. The
576 median survival was significantly increased from 8.9 months to 17.6 months ($p = 0.0001$).
577 Altogether, these results show that the drug delivery using clinically-approved microbubbles,

chemotherapeutics, and ultrasound is feasible and compatible with respect to clinical procedures. Nevertheless, the authors did not provide any evidence that the improved therapeutic efficacy of gemcitabine was related to an increase in intratumoral bioavailability of the drug. In addition, the effects of microbubble-assisted ultrasound treatment alone on the tumor growth were not investigated while recent publications describe that according to the ultrasound parameters, such treatment could induce a significant decrease in tumor volume through a reduction in tumor perfusion as described above.

Hepatic metastases from digestive system

A safety study of chemotherapeutic delivery using microbubble-assisted ultrasound for the treatment of liver metastases from gastrointestinal tumors and pancreatic carcinoma was conducted at Beijing Cancer Hospital, China (Wang, et al. 2018). Thirty minutes after intravenous infusion of chemotherapy (for both monotherapy and combination therapy), 1 mL of SonoVue® microbubbles was intravenously administrated which was repeated another five times in 20 min. An ultrasound probe (C1-5 abdominal convex probe; GE Healthcare, USA) was positioned on the tumor lesion which was exposed to ultrasound at different MIs (0.4 to 1) in contrast mode using a LogiQ E9 scanner (GE Healthcare, USA). The primary aims of this clinical trial were to evaluate the safety of this therapeutic procedure and to explore the largest MI and ultrasound treatment time which cancer patients can tolerate. According to the clinical safety evaluation, all twelve patients showed no serious adverse events. The authors reported that the microbubble mediated-chemotherapy led to fever in two patients. However, there is no clear evidence this related to the microbubble and ultrasound treatment. Indeed, in the absence of direct comparison of these results with a historical group of patients receiving the chemotherapy on its own, one cannot rule out a direct link between the fever and the chemotherapy alone. All the adverse side effects were resolved with symptomatic medication.

In addition, the severity of side effects did not worsen with increases in MI, suggesting that microbubble-mediated chemotherapy is a safe procedure. The secondary aims were to assess the efficacy of this therapeutic protocol using contrast-enhanced CT and MRI. Thus, tumor size and development were characterized according to the RECIST criteria. Half of the patients had stable disease and one patient obtained a partial response after the first treatment cycle. The median progression-free survival was 91 days. However, making any comparison and interpretation of results is very difficult because none of the patients were treated with the same chemotherapeutics, MI, and/or number of treatment cycles. The results of safety and efficacy evaluations should be compared to patients receiving the chemotherapy on its own in order to clearly identify the therapeutic benefit of combining with ultrasound-driven microbubbles. Similar to the pancreatic clinical study, no direct evidence of enhanced therapeutic bioavailability of the chemotherapeutic drug after the treatment was provided. This investigation is all the more important as the ultrasound and microbubble treatment was applied 30 min after intravenous chemotherapy (for both monotherapy and combination therapy) independently of drug pharmacokinetics and metabolism.

Ongoing and upcoming clinical trials

Currently, two clinical trials are ongoing: (i) Prof. F. Kiessling (RWTH Aachen University, Germany) proposes to examine whether the exposure of early primary breast cancer to microbubble-assisted ultrasound during neoadjuvant chemotherapy results in increased tumor regression in comparison to ultrasound treatment alone (NCT03385200); (ii) Dr. J. Eisenbrey (Sidney Kimmel Cancer Center, Thomas Jefferson University, USA) is investigating the therapeutic potential of perflutren protein-type A microspheres in combination with microbubble-assisted ultrasound in radioembolization therapy of liver cancer (NCT03199274).

A proof of concept study (NCT03458975) has been set in Tours Hospital, France for treating non-resectable liver metastases. The aim of this trial is to perform a feasibility study with the development of a dedicated ultrasound imaging and delivery probe with a therapy protocol optimized for patients with hepatic metastases of colorectal cancer and who are eligible for monoclonal antibodies in combination with chemotherapy. A dedicated 1.5D ultrasound probe has been developed and interconnected to a modified Aixplorer® imaging platform (Supersonic imagine, Aix-en-Provence, France). The primary objective of the study is to determine the rate of objective response at two months for lesions receiving optimized and targeted delivery of systemic chemotherapy combining bevacizumab and FOLFIRI compared with those treated with only systemic chemotherapy regimen. The secondary objective is to determine the safety and tolerability of this local approach of optimized intratumoral drug delivery during the three months of follow-up, by assessing tumor necrosis, tumor vascularity and pharmacokinetics of bevacizumab and by profiling cytokine expression spatially.

IMMUNOTHERAPY

Cancer immunotherapy is considered to be one of the most promising strategies to eradicate cancer as it makes use of the patient's own immune system to selectively attack and destroy tumor cells. It is a common name that refers to a variety of strategies that aim to unleash the power of the immune system by either boosting antitumoral immune responses or flagging tumor cells to make them more visible to the immune system. The principle is based on the fact that tumors express specific tumor antigens which are not, or to a much lesser extent, expressed by normal somatic cells and hence can be used to initiate a cancer-specific immune response. In this section we aim to give insight into how microbubbles and ultrasound have

been applied as useful tools to initiate or sustain different types of cancer immunotherapy as illustrated in Figure 3.

When Ralph Steinman (Steinman, et al. 1979) discovered the dendritic cell (DC) in 1973, its central role in the initiation of immunity made it an attractive target to evoke specific antitumoral immune responses. Indeed, these cells very efficiently capture antigens and present them to T-lymphocytes in major histocompatibility complexes (MHCs), thereby bridging the innate and adaptive immune system. More specifically, exogenous antigens engulfed via the endolysosomal pathway are largely presented to $CD4^+$ T cells *via* MHC-II, whereas endogenous, cytoplasmic proteins are shuttled to MHC-I molecules for presentation to $CD8^+$ cells. As such, either $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cell responses are induced. The understanding of this pivotal role played by DCs formed the basis for DC-based vaccination, where a patient's DCs are isolated, modified *ex vivo* to present tumor antigens and re-administered as a cellular vaccine. DC-based therapeutics, however, suffer from a number of challenges, of which the expensive and lengthy *ex vivo* procedure for antigen-loading and activation of DCs is the most prominent (Santos and Butterfield 2018). In this regard, microbubbles have been investigated for direct delivery of tumor antigens to immune cells *in vivo*. Bioley et al. (2015) showed that intact microbubbles are rapidly phagocytosed by both murine and human DCs, resulting in rapid and efficient uptake of surface-coupled antigens without the use of ultrasound. Subcutaneous injection of microbubbles loaded with the model antigen ovalbumin (OVA) resulted in the activation of both $CD8^+$ and $CD4^+$ T cells. Effectively, these T-cell responses could partially protect vaccinated mice against an OVA-expressing Listeria infection. Dewitte et al. (2014) investigated a different approach, making use of messenger RNA (mRNA) loaded microbubbles combined with ultrasound to transfect DCs. As such, they were able to deliver mRNA encoding both tumor antigens as well as immunomodulating molecules directly to the cytoplasm of the DCs. As a result, preferential

presentation of antigen fragments in MHC-I complexes was ensured, favoring the induction of $CD8^+$ cytotoxic T cells. In a therapeutic vaccination study in mice bearing OVA-expressing tumors, injection of mRNA-sonoporated DCs caused a pronounced slowdown of tumor growth and induced complete tumor regression in 30% of the vaccinated animals. Interestingly, in humans, intradermally injected microbubbles have been used as sentinel lymph node detectors as they can easily drain from peripheral sites to the afferent lymph nodes (Sever, et al. 2012a, Sever, et al. 2012b). Since lymph nodes are the primary sites of immune induction, the interaction of microbubbles with intranodal DCs, could be of high value. To this end, Dewitte *et al.* (2015) showed that mRNA-loaded microbubbles were able to rapidly and efficiently migrate to the afferent lymph nodes after intradermal injection in healthy dogs. Unfortunately, further translation of this concept to an *in vivo* setting is not straightforward, as it prompts the use of less accessible large animal models (*e.g.*, pigs, dogs). Indeed, conversely to what has been reported in humans, lymphatic drainage of subcutaneously injected microbubbles is very limited in the small animal models typically used in preclinical research (mice and rats), which is the result of substantial difference in lymphatic physiology.

Another strategy in cancer immunotherapy is adoptive cell therapy, where *ex vivo* manipulated immune effector cells, mainly T cells and NK (natural killer) cells, are employed to generate a robust and selective anticancer immune response (Yee 2018, Hu, et al. 2019). These strategies have mainly led to successes in hematological malignancies, not only because of the availability of selective target antigens, but also because of the accessibility of the malignant cells (Khalil, et al. 2016, Yee 2018). By contrast, in solid tumors, and especially in brain cancers, inadequate homing of cytotoxic T cells or NK cells to the tumor proved to be one of the main reasons for the low success rates, making the degree of tumor infiltration an important factor in disease prognosis (Childs and Carlsten 2015, Gras Navarro, et al. 2015, Yee 2018). To address this, focused ultrasound and microbubbles have been used to make tumors

more accessible to cellular therapies. The first demonstration of this concept was provided by Alkins et al. (2013) who used a xenograft HER-2-expressing breast cancer brain metastasis model to determine whether ultrasound and microbubbles could allow intravenously infused NK cells to cross the blood-brain barrier (BBB). By loading the NK cells with superparamagnetic iron oxide (SPIO) nanoparticles, the accumulation of NK cells in the brain could be tracked and quantified via MRI. An enhanced accumulation of NK cells was found when the cells were injected immediately prior to BBB disruption. Importantly NK cells retained their activity and ultrasound treatment resulted in a sufficient NK to tumor cell ratio to allow effective tumor cell killing (Alkins, et al. 2016). In contrast, very few NK cells reached the tumor site when BBB disruption was absent or performed before NK cell infusion. Although it is not known for certain why timing had such a significant impact on NK extravasation, it is likely that the most effective transfer to the tissue occurs at the time of insonification, and that the barrier is most open during this time (Marty, et al. 2012). Possible other explanations include the difference in size of the temporal BBB openings or a possible alternation in the expression of specific leukocyte adhesion molecules by the BBB disruption, thus facilitating the translocation of NK cells. Also for tumors where BBB crossing is not an issue, ultrasound has been used to improve delivery of cellular therapeutics. Sta Maria et al. (2015) demonstrated enhanced tumor infiltration of adoptively transferred NK cells after treatment with microbubbles and low dose focused ultrasound. This result was confirmed by Yang *et al.* (2019a) in a more recent publication where the homing of NK cells was more than doubled after microbubble injection and ultrasound treatment of an ovarian tumor. Despite the enhanced accumulation, however, the authors did not observe an improved therapeutic effect, which might be due to the limited number of treatments that were applied, or the immunosuppressive tumor microenvironment that counteracts the cytotoxic action of the NK cells.

There is growing interest in exploring the effect of microbubbles and ultrasound on the tumor microenvironment, as recent work has shown that BBB disruption with microbubbles and ultrasound may induce sterile inflammation. Although a strong inflammatory response may be detrimental in the case of drug delivery across the BBB, it might be interesting to further study this inflammatory response in solid tumors as it might induce the release of damage-associated molecular patterns (DAMPS) such as heat-shock proteins and inflammatory cytokines. This could shift the balance towards a more inflammatory microenvironment that could promote immunotherapeutic approaches. As reported by Liu *et al.* (2012) exposure of a CT26 colon carcinoma xenograft to microbubbles and low pressure pulsed ultrasound increased cytokine release and triggered lymphocyte infiltration. Similar data have been reported by Hunt *et al.* (2015). In their study, ultrasound treatment caused a complete shut-down of tumor vasculature followed by the expression of HIF-1α (hypoxia-inducible factor 1α), a marker of tumor ischemia and tumor necrosis, as well as increased infiltration of T cells. Similar responses have been reported following thermal and mechanical HIFU treatments of solid tumors (Unga and Hashida 2014, Silvestrini, et al. 2017). A detailed review of ablative ultrasound therapies is however out of the scope of this review.

At present, the most successful form of immunotherapy is the administration of monoclonal antibodies to inhibit regulatory immune checkpoints that block T cell action. Examples are CTLA-4 (cytotoxic T lymphocyte-associated protein-4) and PD-1 (programmed cell death-1), which act as brakes on the immune system. Blocking the effect of these brakes can revive and support the function of immune effector cells. Despite the numerous successes achieved with checkpoint inhibitors, responses have been quite heterogeneous as the success of checkpoint inhibition therapy largely depends on the presence of intratumoral effector T cells (Weber 2017). This motivated Bulner et al. (2019) to explore the synergy of microbubble and ultrasound treatment with PD-L1 checkpoint inhibition therapy in mice. Tumors in the

treatment group that received the combination of microbubble and ultrasound treatment with checkpoint inhibition were significantly smaller than tumors in the monotherapy groups. One mouse showed complete tumor regression and remained tumor free upon rechallenge, indicative of an adaptive immune response.

Overall, the number of studies that investigate the impact of microbubble and ultrasound treatment on immunotherapy is limited, making this a rather unexplored research area. It is obvious that more in-depth research is warranted to improve our understanding on how (various types of) immunotherapy might benefit from (various types of) ultrasound treatment.

BLOOD BRAIN BARRIER (BBB) AND BLOOD SPINAL CORD BARRIER (BSCB) OPENING

The barriers of the central nervous system (CNS), the Blood-Brain Barrier (BBB) and Blood-Spinal Cord Barrier (BSCB), greatly limit drug-based treatment of CNS disorders. These barriers help to regulate the specialized CNS environment by limiting the passage of most therapeutically relevant molecules (Pardridge 2005). Although several methods have been proposed to circumvent the BBB and BSCB, including chemical disruption and the development of molecules engineered to capitalize on receptor-mediated transport (so-called Trojan Horse molecules), the use of ultrasound in combination with microbubbles (Hynynen, et al. 2001) or droplets (Wu, et al. 2018) to transiently modulate these barriers has come to the forefront in recent years due to the targeted nature of this approach and its ability to facilitate delivery of a wide range of currently available therapeutics. First demonstrated in 2001 (Hynynen, et al. 2001), ultrasound-mediated BBB opening has been the topic of several hundred original research articles in the last two decades, and in recent years has made headlines for ground-breaking clinical trials targeting brain tumors and Alzheimer's disease as described below in the clinical studies section.

Mechanisms, Bioeffects, and Safety

Ultrasound in combination with microbubbles can produce permeability changes in the BBB via both enhanced paracellular and transcellular transport (Sheikov, et al. 2004, Sheikov, et al. 2006). Reduction and reorganization of tight junction proteins (Sheikov, et al. 2008) and upregulation of active transport protein Caveolin-1 (Deng, et al. 2012) have been reported. Although the exact physical mechanisms driving these changes are not known, there are several factors that are hypothesized to contribute to these effects, including direct tensile stresses due to the expansion and contraction of the bubbles in the lumen, as well as shear stresses at the vessel wall arising from acoustic microstreaming. Recent studies have also investigated the suppression of efflux transporters following ultrasound exposure with microbubbles. A reduction in P-glycoprotein expression (Cho, et al. 2016, Aryal, et al. 2017) and BBB transporter gene expression (McMahon, et al. 2018) has been observed by multiple groups. One study showed that P-glycoprotein expression was suppressed for over 48 h following treatment with ultrasound and microbubbles (Aryal, et al. 2017). However, the degree of inhibition of efflux transporters as a result of ultrasound with microbubbles may be insufficient to prevent efflux of some therapeutics (Goutal, et al. 2018), and thus this mechanism requires further study.

Many studies have documented enhanced CNS tumor response following ultrasound and microbubble-mediated delivery of drugs across the Blood-Tumor-Barrier in rodent models. Improved survival has been shown in both primary (Chen, et al. 2010, Aryal, et al. 2013) and metastatic tumor models (Park, et al. 2012, Alkins, et al. 2016).

Beyond simply enhancing drug accumulation in the CNS, several positive bioeffects of ultrasound and microbubble induced BBB opening have been reported. In rodent models of Alzheimer's disease, numerous positive effects have been discovered in the absence of

exogenous therapeutics. These effects include a reduction in amyloid-β plaque load (Jordão, et al. 2013, Burgess, et al. 2014, Leinenga and Götz 2015, Poon, et al. 2018), reduction in tau pathology (Pandit, et al. 2019), and improvements in spatial memory (Burgess, et al. 2014, Leinenga and Götz 2015). Two-photon microscopy has shown that amyloid-β plaque size is reduced in transgenic mice for up to two weeks post ultrasound and microbubble treatment (Poon, et al. 2018). Opening of the BBB in both transgenic and wild-type mice has also revealed enhanced neurogenesis (Burgess, et al. 2014, Scarcelli, et al. 2014, Mooney, et al. 2016) in the treated tissue.

Gene delivery to the CNS using ultrasound and microbubbles is another area that is increasingly being investigated. Viral (Alonso, et al. 2013, Wang, et al. 2015b) and non-viral (Mead, et al. 2016) delivery methods have been investigated. While early studies demonstrated the feasibility of gene delivery using reporter genes (for example Thevenot et al. (2012), Alonso et al. (2013)), there have been promising results delivering therapeutic genes. In particular, advances have been made in Parkinson's disease models, where therapeutic genes have been tested (Mead, et al. 2017, Xhima, et al. 2018), and where long lasting functional improvements have been reported in response to therapy (Mead, et al. 2017). It is expected that research into this highly promising technique will expand to a range of therapeutic applications.

Despite excellent safety profiles in non-human primate studies investigating repeat opening of the BBB (McDannold, et al. 2012, Downs, et al. 2015), there has been recent controversy due to reports of a sterile inflammatory response observed in rats (Kovacs, et al. 2017a, Kovacs, et al. 2017b, Silburt, et al. 2017). The inflammatory response is proportional to the magnitude of BBB opening and is therefore strongly influenced by experimental conditions such as microbubble dose and acoustic settings. However, McMahon and Hynynen (2017) showed that when clinical microbubble doses are used, and treatment exposures are actively controlled to avoid over treating, the inflammatory response is acute and mild. They note that while chronic

inflammation is undesirable, acute inflammation may actually contribute to some of the positive bioeffects that have been observed. For example, the clearance of amyloid-β following ultrasound and microbubble treatment is thought to be mediated in part by microglial activation (Jordão, et al. 2013). These findings reiterate the need for carefully controlled treatment exposures to select for desired bioeffects.

Cavitation Monitoring and Control

It is generally accepted that the behavior of the microbubbles in the ultrasound field is predictive, to an extent, of the observed bioeffects. In the seminal study on the association between cavitation and BBB opening, McDannold et al. (2006) observed an increase in second harmonic emissions in cases of successful opening, compared to exposures that lead to no observable changes in permeability as measured by contrast enhanced MRI. Further, they noted that successful opening could be achieved in the absence of inertial cavitation, which was also reported by another group (Tung, et al. 2010). These general guidelines have been central to the development of active treatment control schemes that have been developed to date – all with the common goal of promoting stable bubble oscillations, while avoiding violent bubble collapse that can lead to tissue damage. These methods are based either on detection of sub or ultraharmonic (O'Reilly and Hynynen 2012, Tsai, et al. 2016, Bing, et al. 2018), harmonic bubble emissions (Arvanitis, et al. 2012, Sun, et al. 2017) or a combination thereof (Kamimura, et al. 2019). An approach based on the sub/ultraharmonic controller developed by O'Reilly and Hynynen (2012) has been employed in early clinical testing (Lipsman, et al. 2018, Mainprize, et al. 2019).

Control methods presented to date have generally been developed using single receiver elements, which simplifies data processing but does not allow signals to be localized. Focused receivers are spatially selective but can miss off-target events, while planar receivers may

generate false positives based on signals originating outside the treatment volume. The solution to this is to use an array of receivers and passive beamforming methods, combined with phase correction methods to compensate for the skull bone (Jones, et al. 2013, 2015) to generate maps of bubble activity. In the brain this has been achieved with linear arrays (Arvanitis, et al. 2013, Yang, et al. 2019c), which suffer from poor axial resolution when using passive imaging methods, as well as large-scale sparse hemispherical or large aperture receiver arrays (O'Reilly, et al. 2014, Deng, et al. 2016, Crake, et al. 2018, Jones, et al. 2018, Liu, et al. 2018a) that optimize spatial resolution for a given frequency. Recently, this has extended beyond just imaging the bubble activity to incorporate real-time, active feedback control based on both the spectral and spatial information obtained from the bubble maps (Jones, et al. 2018) (Figure 4). Robust control methods building on these works will be essential for widespread adoption of this technology to ensure safe and consistent treatments.

BSCB opening

Despite the similarities between the BBB and BSCB, and the great potential benefit for patients, there has been limited work investigating translation of this technology to the spinal cord. Opening of the BSCB in rats was first reported by Wachsmuth et al. (2009), and was followed by studies from Weber-Adrien et al. (2015), Payne et al. (2017), and O'Reilly et al. (2018) in rats (Figure 5) and from Montero et al. (2019) in rabbits, the latter performed through a laminectomy window. In 2018, O'Reilly et al. (2018) presented the first evidence of a therapeutic benefit in a disease model, showing improved tumor control in a rat model of leptomeningeal metastases.

Although promising, there remains significant work to be done to advance BSCB opening to clinical studies. A more thorough characterization of the bioeffects in the spinal cord and how, if at all, they differ from the brain is necessary to ensure safe translation. Additionally,

methods and devices capable of delivering controlled therapy to the spinal cord at clinical scale are needed. While laminectomy and implantation of an ultrasound device (Montero, et al. 2019) might be an appropriate approach for some focal indications, treating multifocal or diffuse disease will require the ultrasound to be delivered through the intact bone to the narrow spinal canal. Fletcher and O'Reilly (2018) have presented a method to suppress standing waves in the human vertebral canal. Combined with devices suited to the spinal geometry, such as that presented by Xu and O'Reilly (2019), these methods will help to advance clinical translation.

Clinical studies

The feasibility of enhancing BBB permeability in and around brain tumors using ultrasound and microbubbles has now been demonstrated in two clinical trials. In the study conducted at Assistance Publique–Hôpitaux de Paris in Paris, France, an unfocused 1 MHz ultrasound transducer (SonoCloud®) was surgically placed over the tumor-resection area and permanently fixed into the hole in the skull bone. The skin was placed over the transducer and after healing, treatments were conducted by inserting a needle probe through the skin to provide the driving signal to the transducer. Monthly treatments were then conducted while infusing a chemotherapeutic agent into the blood stream (carboplatin). The sonication was executed during infusion of SonoVue® microbubbles. A constant pulsed sonication was applied during each treatment followed by a contrast enhanced MRI to estimate BBB permeability. The power was escalated for each monthly treatment until enhancement was detected in MRI. This study demonstrated feasibility and safety (Carpentier, et al. 2016) and a follow up study may indicate increase in survival (Idbaih, et al. 2019).

The second brain tumor study was conducted at Sunnybrook Health Sciences Centre in Toronto, Canada, which used the InSightec Exablate 220 kHz device and through-skull MRI–guided sonications of brain tumors prior to the surgical resection. It also showed the feasibility

of inducing highly localized BBB permeability enhancement, safety, and that chemotherapeutic concentration in the sonicated peritumor tissue was higher than in the unsonicated tissue (Mainprize, et al. 2019).

Another study conducted in Alzheimer's disease patients with the Exablate device demonstrated safe BBB permeability enhancement and that the treatment could be repeated one month later without any imaging or behavior indications of adverse events (Lipsman, et al. 2018). A third study with the same device investigated the feasibility of using functional MRI to target motor cortex in Amyotrophic Lateral Sclerosis (ALS) patients again showing precisely targeted BBB permeability enhancement without adverse effects in this delicate structure (Abrahao, et al. 2019). All of these studies were conducted using Definity® microbubbles. These studies have led to the current ongoing brain tumor trial with six monthly treatments of the brain tissue surrounding the resection cavity during the maintenance phase of the treatment with temozolomide. This study sponsored by InSightec is being conducted in multiple institutions. Similarly, a phase II trial in Alzheimer's disease sonicating the hippocampus with the goal of investigating the safety and potential benefits from repeated (three treatments with two-week interval) BBB permeability enhancement alone is ongoing. This study is also being conducted in several institutions that have the device.

SONOTHROMBOLYSIS

Occlusion of blood flow through diseased vasculature is caused by thrombi, blood clots which form in the body. Due to limitations in thrombolytic efficacy and speed, sonothrombolysis, ultrasound which accelerates thrombus breakdown alone, or in combination with thrombolytic drugs and/or cavitation nuclei, has been under extensive investigation in the last two decades (Bader, et al. 2016). Sonothrombolysis promotes thrombus dissolution for the treatment of stroke (Alexandrov, et al. 2004a, Alexandrov, et al. 2004b, Molina, et al. 2006,

Chen, et al. 2019), myocardial infarction (Mathias, et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019), acute peripheral arterial occlusion (Ebben, et al. 2017), deep vein thrombosis (Shi, et al. 2018), and pulmonary embolism (Dumantepe, et al. 2014, Engelberger and Kucher 2014, Lee, et al. 2017).

Mechanisms, Agents, and Approaches

Ultrasound improves recombinant tissue plasminogen activator (rt-PA) diffusion into thrombi and augments lysis primarily via acoustic radiation force and streaming (Datta, et al. 2006, Prokop, et al. 2007, Petit, et al. 2015). Additionally, ultrasound increases rt-PA and plasminogen penetration into the thrombus surface and enhances removal of fibrin degradation products via ultrasonic bubble activity, or acoustic cavitation, that induces microstreaming (Elder 1958, Datta, et al. 2006, Sutton, et al. 2013). Two types of cavitation are correlated with enhanced thrombolysis: stable cavitation, with highly nonlinear bubble motion resulting in acoustic emissions at the subharmonic and ultraharmonics of the fundamental frequency (Flynn 1964, Phelps and Leighton 1997, Bader and Holland 2013), and inertial cavitation, with substantial radial bubble growth and rapid collapse generating broadband acoustic emissions (Carstensen and Flynn 1982, Flynn 1982).

Specialized contrast agents and tailored ultrasound schemes have been investigated with the aim of optimizing sonothrombolysis. Petit et al. (2015) observed a greater degree of rt-PA lysis with BR38 microbubbles exposed to 1 MHz pulsed ultrasound at an amplitude causing inertial cavitation (1.3 MPa peak rarefactional pressure) than at a lower amplitude causing stable cavitation (0.35 MPa peak rarefactional pressure). Goyal et al. (2017) also measured a higher degree of thrombolysis with 1 MHz pulsed ultrasound at 1.0 MPa peak rarefactional pressure with inertial cavitation than at 0.23 MPa peak rarefactional pressure with stable cavitation in an *in vitro* model of microvascular obstruction using perfluorobutane-filled, lipid

shelled microbubbles (Weller, et al. 2002) as a nucleation agent. However, Kleven et al. (2019) observed more than 60% fractional clot width loss for highly retracted human whole blood clots exposed to rt-PA, Definity® and 220 kHz pulsed or continuous wave (CW) ultrasound at an acoustic output with sustained stable cavitation throughout the insonification periods (0.22 MPa peak rarefactional pressure) (Figure 6).

Echogenic liposomes loaded with rt-PA enhanced lysis compared to rt-PA alone at concentrations of 1.58 and 3.15 mg/mL (Shekhar, et al. 2017), suggesting that encapsulation of rt-PA could reduce the rt-PA dose by a factor of two with equivalent lytic activity. Subsequently it has been demonstrated that these liposomes protect rt-PA against degradation by plasminogen activator inhibitor-1 (PAI-1), while achieving equivalent thrombolytic efficacy relative to rt-PA, Definity®, and intermittent 220 kHz CW ultrasound (Shekhar, et al. 2019). Promising agents, including a nanoscale (< 100 nm) contrast agent (Brüssler, et al. 2018) and magnetically targeted microbubbles (De Saint Victor, et al. 2019), have also demonstrated enhanced rt-PA thrombolysis *in vitro*. All of these investigators noted that in the absence of rt-PA, the combination of ultrasound and microbubbles did not degrade the fibrin network.

Several minimally invasive techniques have also been explored, with or without the inclusion of rt-PA or exogenous cavitation nuclei. In the clinical management of stroke, rapid treatments are needed because of the neurologist's adage "time is brain". Thus, treatment options that promote fast clot removal, reduce edema and intracerebral bleeding, and improve patient outcomes are of immense value. Magnetic resonance image-guided high intensity focused ultrasound has been investigated for the treatment of both ischemic (Burgess, et al. 2012) and hemorrhagic (Monteith, et al. 2013) stroke, and Zafar et al. (2019) have provided an excellent review of the literature for this approach. Histotripsy, a form of high intensity focused ultrasound that relies on the mechanical action of microbubble clouds to ablate thrombi with

and without rt-PA (Maxwell, et al. 2009, Bader, et al. 2015, Zhang, et al. 2016b, Bader, et al. 2019) is under development to treat deep vein thrombosis. Additionally, ultrasound-accelerated catheter-directed thrombolysis using the EKOS system (EKOS/BTG, Bothell, WA, USA) combines 2 MHz low-intensity pulsed ultrasound and rt-PA without cavitation nuclei to improve lytic efficiency to treat deep vein thrombosis (Shi, et al. 2018) and pulmonary embolism (Garcia 2015).

Cavitation monitoring

Acoustic cavitation has been shown to mediate direct fibrinolysis (Weiss, et al. 2013) and accelerated rt-PA lysis (Everbach and Francis 2000, Datta, et al. 2006, Prokop, et al. 2007, Hitchcock, et al. 2011). Passive and active cavitation detection techniques have been developed to monitor acoustic cavitation (Roy, et al. 1990, Madanshetty, et al. 1991, Bader, et al. 2015). Passive cavitation imaging, or passive acoustic mapping, employs a transducer array that listens passively (i.e., no transmit) to emissions from acoustically activated microbubbles (Salgaonkar, et al. 2009, Gyöngy and Coussios 2010, Haworth, et al. 2017). Vignon et al. (2013) developed a prototype array enabling spectral analysis of bubble activity for sonothrombolysis applications. Superharmonic Doppler effects have also been utilized to monitor bubble activity from 500 kHz pulsed therapeutic ultrasound (Pouliopoulos and Choi 2016). Both a linear array (Arvanitis and McDannold 2013a, Arvanitis, et al. 2013, Arvanitis and McDannold 2013b) and a sparse hemispherical array (Acconcia, et al. 2017) have been integrated into a clinical magnetic resonance image-guided high intensity focused ultrasound system to assess microbubble dynamics during sonothrombolysis in the brain.

Preclinical studies

Information gathered from animal studies can help inform human clinical trials, despite a strong species dependence of clot rt-PA lytic susceptibility (Gabriel, et al. 1992, Flight, et al. 2006, Huang, et al. 2017). A comprehensive systematic evaluation of 16 *in vivo* preclinical sonothrombolysis studies was carried out by Auboire et al. (2018) summarizing treatment efficacy and safety outcomes in models of ischemic stroke. Since that review was published, the efficacy of sonothrombolysis using nitrogen microbubbles stabilized with a non-crosslinked shell delivered intra-arterially through a catheter and rt-PA delivered intravenously has been demonstrated in a rat model of ischemic stroke (Dixon, et al. 2019).

Clinical studies

A rich literature exists of clinical trials exploring the safety and efficacy of sonothrombolysis. Two recent meta-analyses of seven randomized controlled trials (Chen, et al. 2019, Zafar, et al. 2019) attempt to determine whether the administration of rt-PA and ultrasound improve outcomes in acute ischemic stroke. Both analyses conclude that sonothrombolysis significantly enhances complete or partial recanalization, with improved neurologic function (assessed via the National Institutes of Health Stroke Scale, NIHSS). An ongoing clinical trial (TRUST; NCT03519737) will determine whether large vessel occlusions can be recanalized with sonothrombolysis (Cerevast Medical, Inc., Bothell, WA, USA) and rt-PA, tenecteplase or alteplase, (Campbell, et al. 2018) while patients are transferred to a stroke center for mechanical thrombectomy (Gauberti 2019).

Several clinical trials have shown that high MI pulsed diagnostic ultrasound exposure of Definity® before and after percutaneous coronary intervention for ST elevation myocardial infarction can prevent microvascular obstruction and improve functional outcomes (Mathias, et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019). A systematic review of 16 catheter-directed sonothrombolysis clinical trials comprised mostly of retrospective case series using

the EKOS system without microbubble infusions determined that this treatment modality is safe and promising for the treatment of deep vein thrombosis, DVT (Shi, et al. 2018). However, a large-sample randomized prospective clinical trial is needed to improve the clinical evidence for use as a front-line therapy for DVT. In retrospective studies in patients with pulmonary embolism Lee et al. (2017) conclude that catheter directed sonothrombolysis is safe and decreases right-sided heart strain, but Schissler et al. (2018) conclude that this therapy is not associated with a reduction in mortality nor increased resolution of right ventricular dysfunction. And finally, an ongoing trial in a small cohort of 20 patients with acute peripheral arterial occlusions (Ebben, et al. 2017) will determine whether Luminity® (marketed in the US as Definity®) and 1.8 MHz transdermal diagnostic ultrasound with intermittent high MI (1.08) and low MI (0.11) for visualization of the microbubbles and flow will improve recanalization. In summary, sonothrombolysis has demonstrated clinical benefit in the treatment of acute and chronic thrombotic disease. Ultrasound-assisted thrombolysis has a potential role as an emerging viable and therapeutic option for future management of stroke and cardiovascular disease.

CARDIOVASCULAR DRUG DELIVERY AND THERAPY

In cardiovascular drug delivery, cavitation nuclei are co-administered or loaded with different therapeutics for the treatment of various diseases. For atherosclerosis treatment in an ApoE-deficient mouse model, intercellular adhesion molecule-1 targeted microbubbles carrying angiogenesis inhibitor Endostar were used (Yuan, et al. 2018). Upon intermittent insonification over the abdominal and thoracic cavity with 1 MHz ultrasound (2 W/cm^2 intensity, 50% duty cycle) for 30 s with two repeats and another treatment 48 h later, plaque area and intraplaque neovascularization were significantly reduced two weeks after treatment. Percutaneous coronary intervention is often used to restore blood flow in atherosclerotic

arteries. The treatment of coronary microembolization, a complication of percutaneous coronary intervention, was demonstrated in pigs treated with ultrasound (1 MHz, 2.0 W/cm^2 intensity, 10 s on and 10 s off, 20 min duration) and microRNA-21-loaded microbubbles four days before coronary microembolization (Su, et al. 2015). This resulted in an improved cardiac dysfunction. Although not a therapeutic study, Liu et al. (2015) did show that plasmid transfection to the myocardium was significantly larger when the microbubbles were administered into the coronary artery compared to intravenously via the ear vein in pigs even though the intracoronary microbubble dose was half of the intravenous dose (1 MHz ultrasound, 2 W/cm^2, 50% duty cycle, 20 min duration). Percutaneous coronary intervention can also result in neointimal formation which induces restenosis. Sirolimus-loaded microbubbles were shown to reduce neointimal formation in coronary arteries by 50% in pigs, see Figure 7, 28 days after angioplasty in combination with a mechanically rotating intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative pressure) (Kilroy, et al. 2015). Another research group showed that paclitaxel-loaded microbubbles and ultrasound (1 MHz, 1.5 MPa for 10 s) can also significantly inhibit neointimal formation in the iliac artery in rabbits one week after percutaneous coronary intervention (Zhu, et al. 2016).

In diabetic cardiomyopathy, microbubble-mediated delivery of fibroblast growth factor has shown therapeutic effects. Zhao et al (2016) could prevent diabetic cardiomyopathy in rats by treating the heart with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) and microbubbles co-administered with acidic fibroblast growth factor nanoparticles twice weekly for 12 consecutive weeks. In already established diabetic cardiomyopathy in rats, the same investigators co-administered basic fibroblast growth factor-containing nanoparticles with microbubbles with the same ultrasound treatment, albeit that it was given three times with one day in between treatments. At four weeks after treatment, this resulted in restored cardiac

functions as a result of structural remodeling of the cardiac tissue (Zhao, et al. 2014). Microbubbles loaded with acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) also showed significantly improved cardiac function in a rat model of diabetic cardiomyopathy. Treatment was performed twice weekly for 12 consecutive weeks (Zhang, et al. 2016a). For doxorubicin induced cardiomyopathy, repeated co-administration of microbubbles and nanoparticles containing acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) applied at the heart successfully prevented doxorubicin induced cardiomyopathy in rats (Tian, et al. 2017). Once doxorubicin induced cardiomyopathy had occurred, microbubble-mediated reversal of cardiomyopathy was shown by the delivery of survivin plasmid to cardiomyocytes and endothelial cells (Lee, et al. 2014) or glucagon-like peptide-1 (GLP-1) to cardiomyocytes, endothelial cells, vascular muscle cells, and mesenchymal cells (Chen, et al. 2015) in rats. The ultrasound settings were 5 MHz (120 V power, pulsing interval of 10 cardiac cycles at end-systole) for a 5 min treatment (Lee, et al. 2014) or not specified (Chen, et al. 2015). The microbubble-mediated gene therapy study by Chen et al. (2016) showed that ANGPTL8 gene therapy does not need to be done in the heart to reverse doxorubicin induced cardiomyopathy in rats as their microbubble and ultrasound (1.3 MHz, 1.4 MPa peak negative pressure, four bursts triggered to every fourth end-systole using a delay of 45-70 ms of the peak of the R wave) therapy was done in the liver (90 s treatment). This resulted in overexpression of ANGPTL8 in liver cells and blood which stimulated cardiac progenitor cells in the epicardium.

A few dozen articles have been published on treating myocardial infarction with microbubble and ultrasound-mediated gene delivery *in vivo*, in mouse, rat, rabbit, and dog models. These are reviewed by Qian et al. (2018). Amongst these are a few targeted microbubble studies which all show that the targeted microbubbles induced higher degrees of

gene transfection, increased myocardial vascular density, and improved cardiac function in comparison to non-targeted microbubbles. This improvement occurred independent of the type of ligand on the microbubble, the gene that was transfected, or the animal model: matrix metalloproteinase 2 target with Timp3 gene in rats (Yan, et al. 2014), intracellular adhesion molecule-1 target with Ang-1 gene in rabbits (Deng, et al. 2015), P-selectin target with hVEGF165 gene in rats (Shentu, et al. 2018). Ultrasound settings for these studies were similar at 1.6 MHz (1.6 MPa peak negative pressure, pulsing interval of four cardiac cycles) for 20 min during infusion of the plasmid-loaded microbubbles (both Yan et al. (2014) and Shentu et al. (2018)), or 1.7 MHz (1.7 MPa peak negative pressure, pulsing interval every four to eight cardiac cycles) for 5 min after bolus injection of the plasmid-loaded microbubbles (Deng, et al. 2015).

Other gene therapy studies for vascular disease include stimulating angiogenesis for the treatment of chronic hindlimb ischemia in rats using miR-126-3p-loaded microbubbles and ultrasound (1.3 MHz, 2.1 MPa peak negative acoustic pressure, pulsing interval 5 s). The treatment lasted for 20 min of which microbubbles were infused for 10 min and resulted in improved perfusion, vessel density, arteriolar formation, and neovessel maturation (Cao, et al. 2015). Recently, successful gene therapy was demonstrated in baboons where Vascular Endothelial Growth Factor (VEGF)-plasmid loaded microbubbles were infused and ultrasound (2-6 MHz, MI 1.9, repeated 5 s burst pulses with three bursts per minute) was applied for 10 min on days 25, 35, 45, and 55 of gestation with the transducer placed over the placental basal plate (Babischkin, et al. 2019). This was a mechanistic study elucidating the role of VEGF in uterine artery remodeling.

The gas core of the cavitation nuclei can also be the therapeutic. Sutton et al. (2014) have shown that ultrasound-mediated (1 MHz, 0.34 MPa acoustic pressure, 30 cycle pulse, 50 s treatment) nitric oxide gas delivery from echogenic liposomes to *ex vivo* perfused porcine

carotid arteries induces potent vasorelaxation. The vasodialative effect of nitric oxide-loaded echogenic liposomes upon insonification (5.7 MHz, 0.36 MPa peak negative pressure, 30 s treatment) was also shown in *ex vivo* perfused rabbit carotid arteries with arterial wall penetration of nitric oxide confirmed by fluorescence microscopy (Kim, et al. 2014). In addition to this, vasodialative effects were demonstrated in carotid arteries *in vivo* in rats with vasospasms following subarachnoid hemorrhage using 1 MHz ultrasound with 0.3 MPa peak-to-peak pressure, 50% duty cycle for a duration of 40 min with constant infusion of the echogenic liposomes. This resulted in improved neurological function (limb placement, beam and grid walking) (Kim, et al. 2014). Ultrasound-activation of the antioxidant hydrogen gas encapsulated in microbubbles was shown to prevent myocardial ischemia-reperfusion injury in rats when administered before reperfusion (He, et al. 2017). There was a dose-dependent effect as 2×10^{10} microbubbles resulted in a more significant reduction in infarct size (70%) than 4×10^{9} microbubbles (39%) compared to vehicle-treated rats. Furthermore, treatment with the high dose hydrogen-microbubbles prevented changes in left ventricular end-diastolic and left ventricular end-systolic dimension as well as minimal reductions in ejection fraction and fractional shortening. Histological and ELISA analysis showed a reduced degree of myocardial necrosis, apoptosis, hemorrhaging, inflammation, and oxidant damage. At the same time that cardiovascular drug delivery and therapy using microbubbles and ultrasound is moving forward to large animal and clinical studies, sophisticated *in vitro* models are being used and/or developed for mechanistic studies, such as flow chambers (µSlides, Ibidi) (Shamout, et al. 2015) and perfused 3D microvascular networks (Juang, et al. 2019) in which human umbilical vein endothelial cells are grown.

Clinical study

Microbubbles and ultrasound were clinically investigated to augment muscle blood flow in 12 patients with stable sickle cell disease in the absence of a drug at the Oregon Health & Science University, Portland, Oregon, USA (Belcik, et al. 2017). Perfusion increased ~2-fold in the forearm flexor muscles upon Definity® infusion and insonification at 1.3 MHz (MI 1.3). Ultrasound was applied 3 times for 3 min with ~5 min intervals. The change in perfusion was determined from contrast enhanced ultrasound imaging and extended well beyond the region where ultrasound was applied. This study showed that the therapeutic ultrasound settings directly translate from mouse to man for superficial muscles, as the same investigators demonstrated augmented blood flow in ischemic and non-ischemic hindlimb muscles in mice in the same study and an earlier publication (Belcik, et al. 2015). However, for the preclinical studies custom-made microbubbles were used instead of Definity®.

SONOBACTERICIDE

Sonobactericide has been defined as the use of ultrasound in the presence of cavitation nuclei for the enhancement of bactericidal action (Lattwein, et al. 2018). This topic has recently gained attention with 17 papers being published in the last five years. Research on ultrasound-mediated enhancement of antimicrobials has focused on several sources of infections including general medical devices (Ronan, et al. 2016, Dong, et al. 2017, Dong, et al. 2018, Hu, et al. 2018, Fu, et al. 2019), acne (Liao, et al. 2017), chronic bacterial prostatitis (Yi, et al. 2016), infective endocarditis (Lattwein, et al. 2018), pneumonia (Sugiyama, et al. 2018), prosthetic joint infections (Li, et al. 2015, Lin, et al. 2015, Guo, et al. 2017a, Zhou, et al. 2018), or urinary tract infections (Horsley, et al. 2019). However, there was no specific disease aim in two studies (Zhu, et al. 2014, Goh, et al. 2015). One group targeted membrane biofouling for water and wastewater industries (Agarwal, et al. 2014). Direct bacterial killing, biofilm degradation and dispersal, and increased or synergistic therapeutic effectiveness of antimicrobials have been

reported as the therapeutic effects of sonobactericide. These studies show that sonobactericide can be applied to treat Gram+ or Gram– bacteria, when they are planktonic, associated with a surface and embedded in biofilm, or intracellular. The majority of these studies were carried out *in vitro*. However, seven were performed *in vivo* in either mice (Li, et al. 2015, Liao, et al. 2017, Sugiyama, et al. 2018, Zhou, et al. 2018), rats (Yi, et al. 2016), or rabbits (Lin, et al. 2015, Dong, et al. 2018). Sonobactericide was mostly performed with co-administration of antimicrobials. Investigators also employed an antimicrobial encapsulated in liposomes that were conjugated to the microbubbles (Horsley, et al. 2019), or the antimicrobial lysozyme was a microbubble coating (Liao, et al. 2017), or did not use antimicrobials altogether (Agarwal, et al. 2014, Goh, et al. 2015, Yi, et al. 2016). An extensive review of sonobactericide has been published recently by Lattwein et al. (2019). Although sonobactericide is an emerging strategy to treat bacterial infections with intriguing potential, the mechanism and the safety of the treatment should be explored, particularly regarding biofilm degradation and dispersal. Future studies should also focus on maximizing the efficacy of sonobactericide *in situ*.

FUTURE PERSPECTIVES AND CONCLUSIONS

Therapeutic ultrasound technology is experiencing a paradigm shift in terms of both technical developments and clinical applications. In addition to its inherent advantages for imaging (e.g., real time nature, portability and low cost), ultrasound in combination with cavitation nuclei is under exploration as a drug delivery modality. The results from several preclinical studies have already demonstrated the potential of ultrasound-responsive cavitation nuclei to deliver multiple types of drugs (including model drugs, anticancer, therapeutic antibodies, genes, nanoparticles, etc.) efficiently in various tumor models, including both ectopic and orthotopic models, for immunotherapy, brain disease, to promote the dissolution of clots, and in the treatment of cardiovascular disease and bacterial infections.

Based on these encouraging preclinical data, several clinical trials have been initiated and others are planned. However, whilst animal studies provide proof of concept, and impetus for clinical studies, careful attention must be given to their relevance in human disease; in particular, the applicability of therapeutic protocols, and appropriate ultrasound settings. Otherwise we risk underestimating the therapeutic effects and potential deleterious side effects. The elucidation of all of the interactions between cavitation nuclei – cells and drugs will help to address this need. The biggest challenges lie in the large differences in timescales between the cavitation nuclei, drug release and uptake, and the biological response (Figure 8). A multidisciplinary approach is needed to tackle these challenges integrating expertise in physics, biophysics, biology, chemistry, and pharmacology.

Custom-made microbubbles which serve as cavitation nuclei are often used for ultrasound-mediated drug delivery studies. An advantage is full control over the payload, as well as the disease target. At the same time, full acoustical characterization and sterility of the microbubbles must be considered during translation to human studies, which often requires approval from the United States Food and Drug Administration (FDA) or other similar federal agencies in Europe and Asia. As an example, for gene therapy, will each different type of genetic material loaded onto microbubbles need such approval, or will a class of cationic microbubbles be approved regardless of the specific gene? The former path would hinder fast clinical translation. For now, co-administration of drugs with FDA-approved ultrasound contrast agents is being explored in clinical trials. Apart from applications in the brain, ongoing clinical studies evaluating microbubble-mediated drug delivery are based on standard clinical ultrasound scanners operating mostly in Doppler mode. In order to promote the progress of this emerging technology, it is very important to design and implement specific therapeutic ultrasound pulse sequences that might be vastly different from clinical diagnostic imaging output. Clinical scanners can indeed be modified to be able to generate drug delivery protocols.

In a similar way that elastography requires long ultrasound pulses to generate the push sequences (Deffieux, et al. 2009) , ultrasound scanners can be modified to be able to transmit drug delivery ultrasound sequences with tailored and optimized parameters (pulse duration, duty cycle, and center frequency).

Ultimately, ultrasound image-guided drug delivery and the monitoring of treatment response could be feasible with the same equipment. Additionally, with recent developments in ultrasound imaging technology, ultrasound-mediated therapy could be planned, applied and monitored in a rapid sequence with high spatial and temporal resolution. The use of a single imaging and therapy device would alleviate the need for co-registration, because the imaging equipment would also be used to induce localized therapy ensuring a perfect co-location. Nonetheless, a compromise between efficacy and safety remains a major challenge for successful clinical applications of this dual methodology, which combines real-time image guidance of therapeutic delivery.

In conclusion, ultrasound-responsive microbubbles which serve as cavitation nuclei are being used to treat a wide variety of diseases and show great potential preclinically and clinically. The elucidation of the cavitation nuclei – cell – interaction and the implementation of drug delivery ultrasound sequences on clinical ultrasound scanners are expected to invigorate clinical studies.

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FIGURE CAPTIONS LIST

Figure 1. Combined effect of nonlinear propagation and focusing of the harmonics in a perfluoropentane micrometer-sized droplet. The emitted ultrasound wave has a frequency of 3.5 MHz and a focus at 3.81 cm, and the radius of the droplet is 10 μm for ease of observation. The pressures are given on the axis of the droplet along the propagating direction of the ultrasound wave, and the shaded area indicates the location of the droplet (reprinted with permission from Sphak et al. (2014)).

Figure 2. Ultrasound-activated microbubbles can locally alter the tumor microenvironment through four mechanisms: enhanced permeability, improved contact, reduced hypoxia, and altered perfusion.

Figure 3. Schematic overview of how microbubbles and ultrasound have been shown to contribute to cancer immunotherapy. From left to right: microbubbles can be used as antigen carriers to stimulate antigen uptake by dendritic cells. Microbubbles and ultrasound can alter the permeability of tumors thereby increasing the intratumoral penetration of adoptively transferred immune cells or checkpoint inhibitors. Finally, exposing tissues to cavitating microbubbles can induce sterile inflammation by the local release of DAMPS.

Figure 4. 3D transcranial subharmonic microbubble imaging and treatment control *in vivo* in rabbit brain during BBB opening. Spectral information (top) shows the appearance of subharmonic activity at t = 35 s into the treatment. Passive mapping of the subharmonic band localizes this activity to the target region. Scale bar indicates 2.5 mm (reprinted (adapted) with permission from Jones et al. (2018)).

Figure 5. T_1 weighted sagittal MR images showing leptomeningeal tumors in rat spinal cord (grey arrowheads) before ultrasound and microbubble treatment (left column), and the enhancement of the cord indicating BSCB opening (white arrows) post-ultrasound and microbubble treatment (right column) (reprinted (adapted) with permission from O'Reilly et al. (2018)).

Figure 6. Simulated acoustic pressure and temperature in a representative subject exposed to pulsed 220 kHz ultrasound with a 33.3% duty cycle. The absolute peak-to-peak pressure maximum for the simulations is displayed in gray scale. Temperature is displayed using a heat map with a minimum color priority write threshold of 1 °C. Computed tomography features such as bone (cyan), skin and internal epithelium (beige), and clot (green), are plotted using contour lines. The transducer is outlined in magenta. Constructive interference is prominent in the soft tissue between the temporal bone and the transducer. Some constructive interference is also present in the brain tissue close to the contralateral temporal bone, however, the pressure in this region did not exceed the pressure in the M1 section of the middle cerebral artery. Temperature rise was prominent in the ipsilateral bone along the transducer axis. Computational model is described in Kleven et al. (2019).

Figure 7. Histological sections of a coronary artery of a pig 28 days after angioplasty. Pigs were treated with sirolimus-loaded microbubbles only (a) or sirolimus-loaded microbubbles and ultrasound (b) using a mechanically rotating intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative pressure). Treatment with ultrasound and sirolimus-loaded microbubbles reduced neointimal formation by 50%. In both sections the intima (I) and media (M) are outlined; scale bar is 500 μm (Reprinted by permission from Springer Nature: Springer, Annals of Biomedical Engineering, Reducing Neointima Formation in a Swine Model with IVUS and Sirolimus Microbubbles, Kilroy JP, Dhanaliwala AH, Klibanov AL, Bowles DK, Wamhoff BR, Hossack JA, COPYRIGHT (2015)).

Figure 8. Different time scales of the therapeutic effects of ultrasound and cavitation nuclei treatment. $[Ca^{2+}]_i$ = intracellular calcium; ROS = reactive oxygen species; ATP = adenosine

triphosphate; EV = extracellular vesicles (reprinted (adapted) with permission from Lattwein et al. (2019)).

Figure 1

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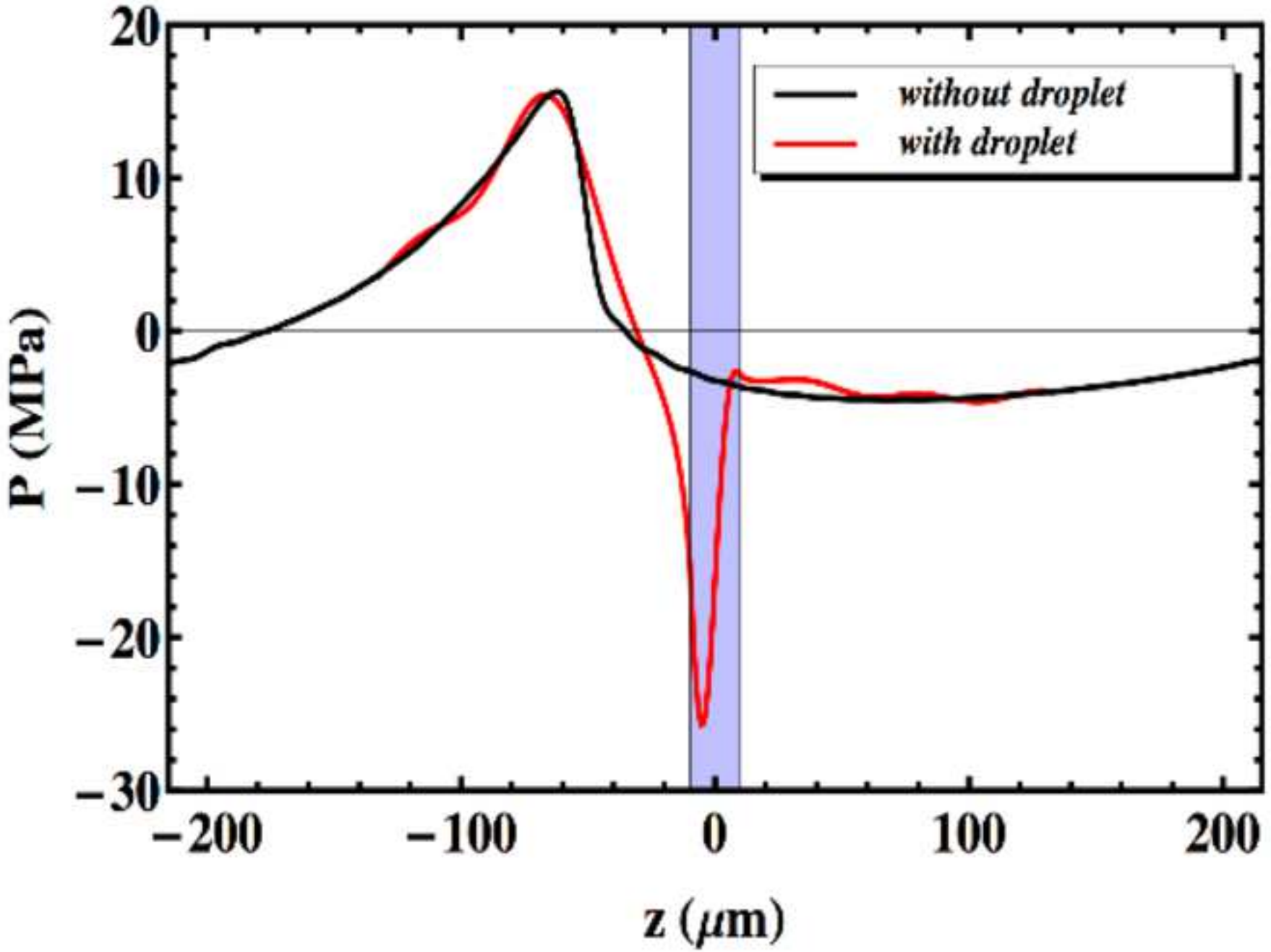


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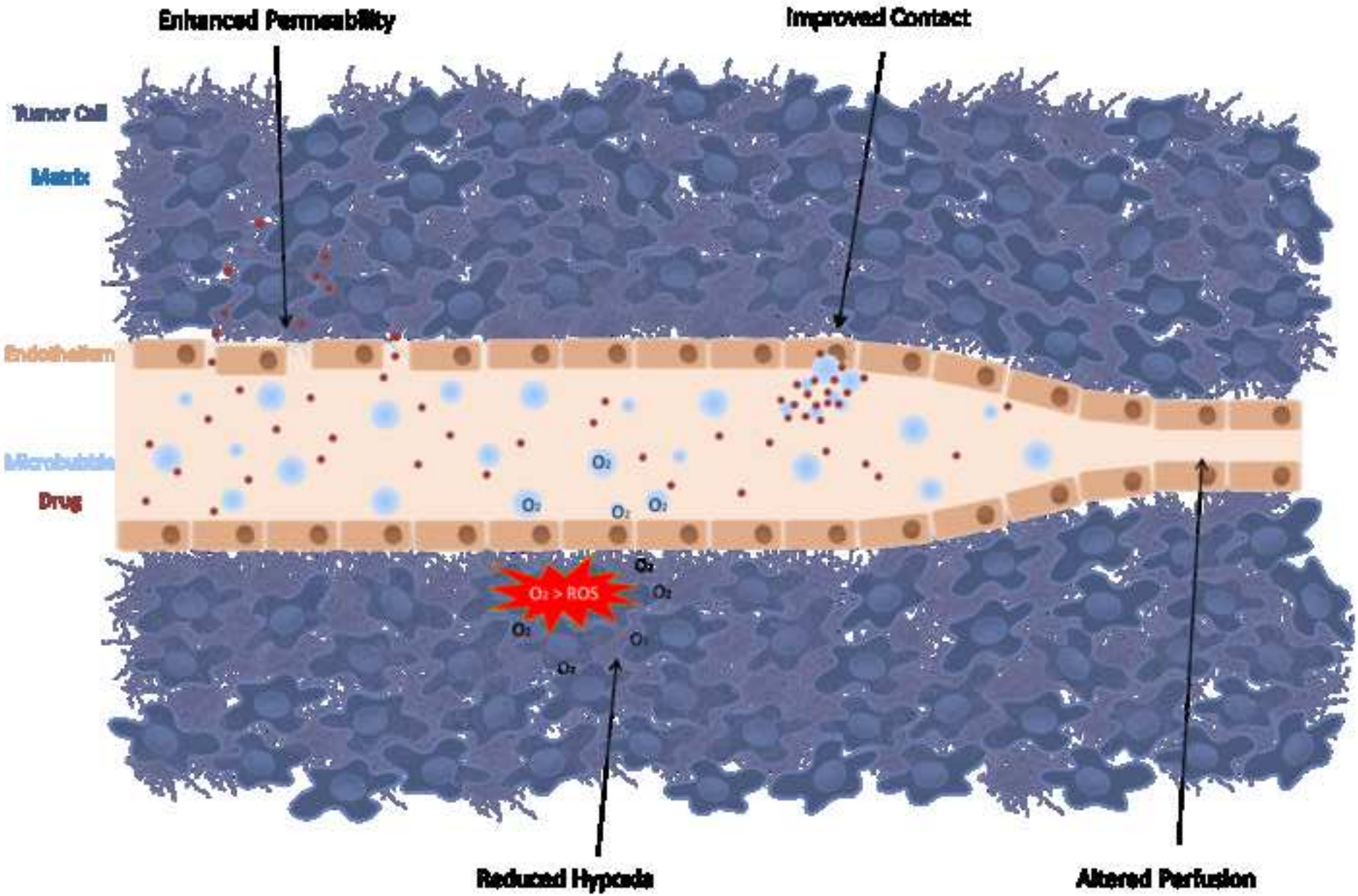


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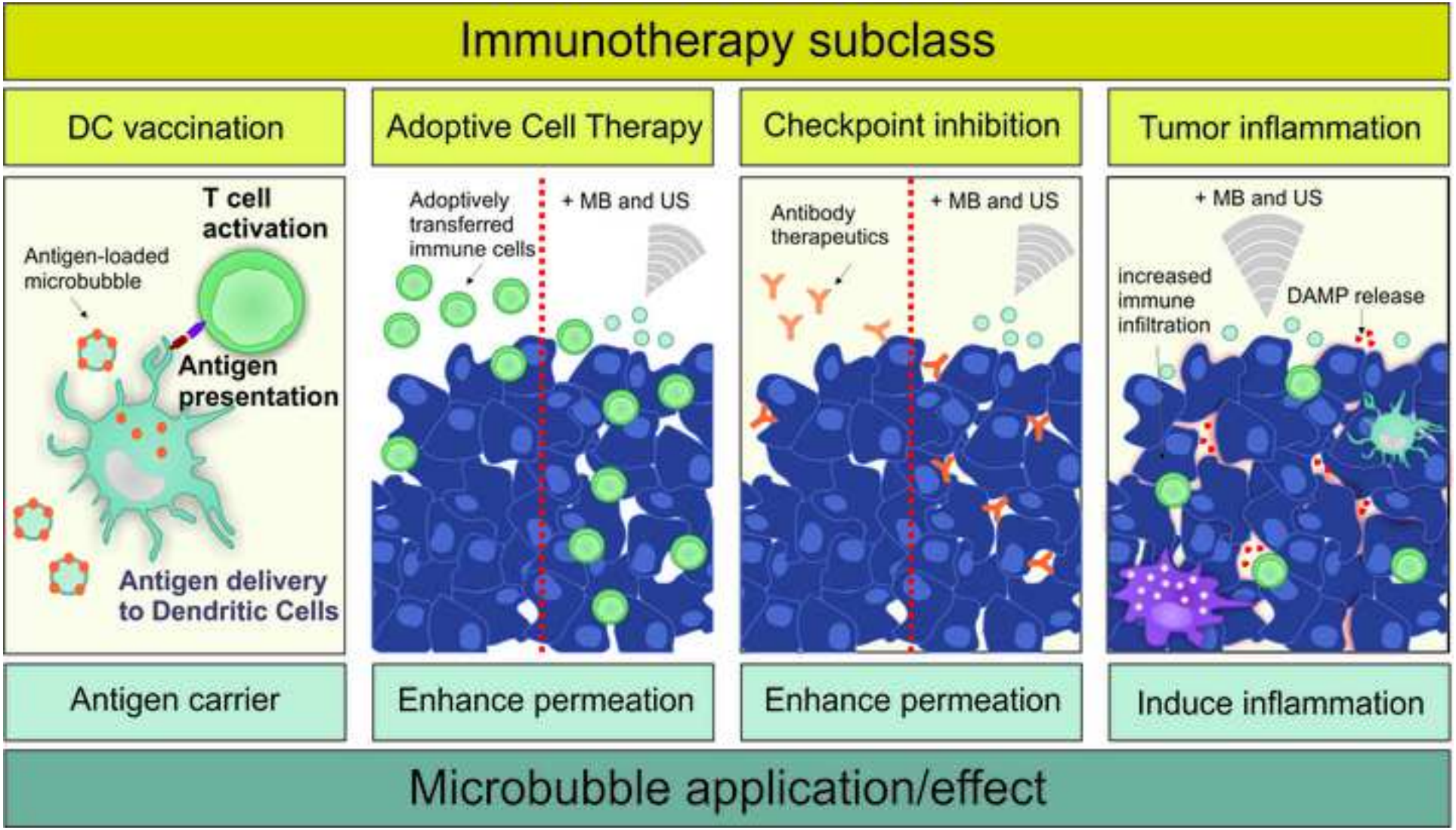


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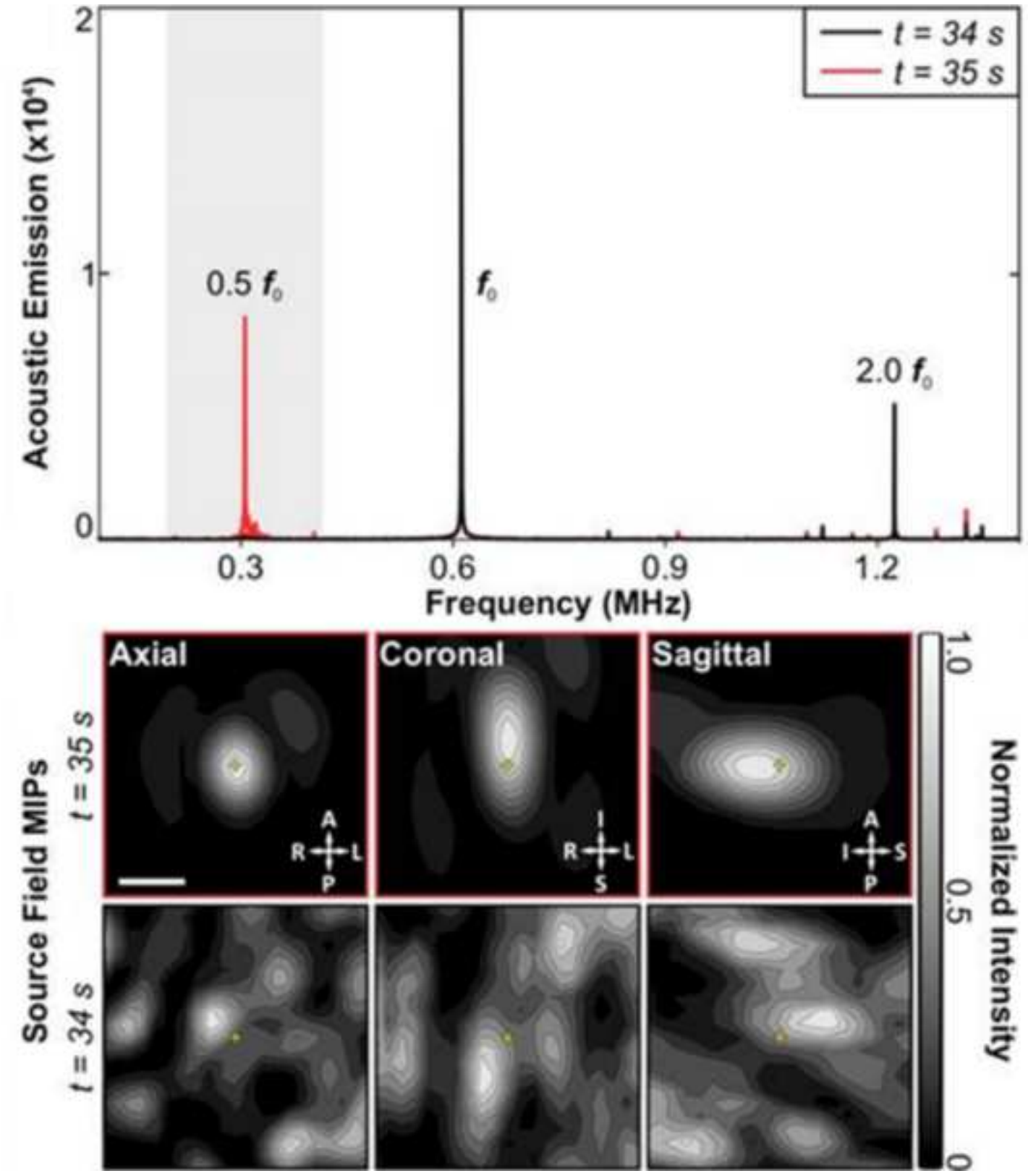


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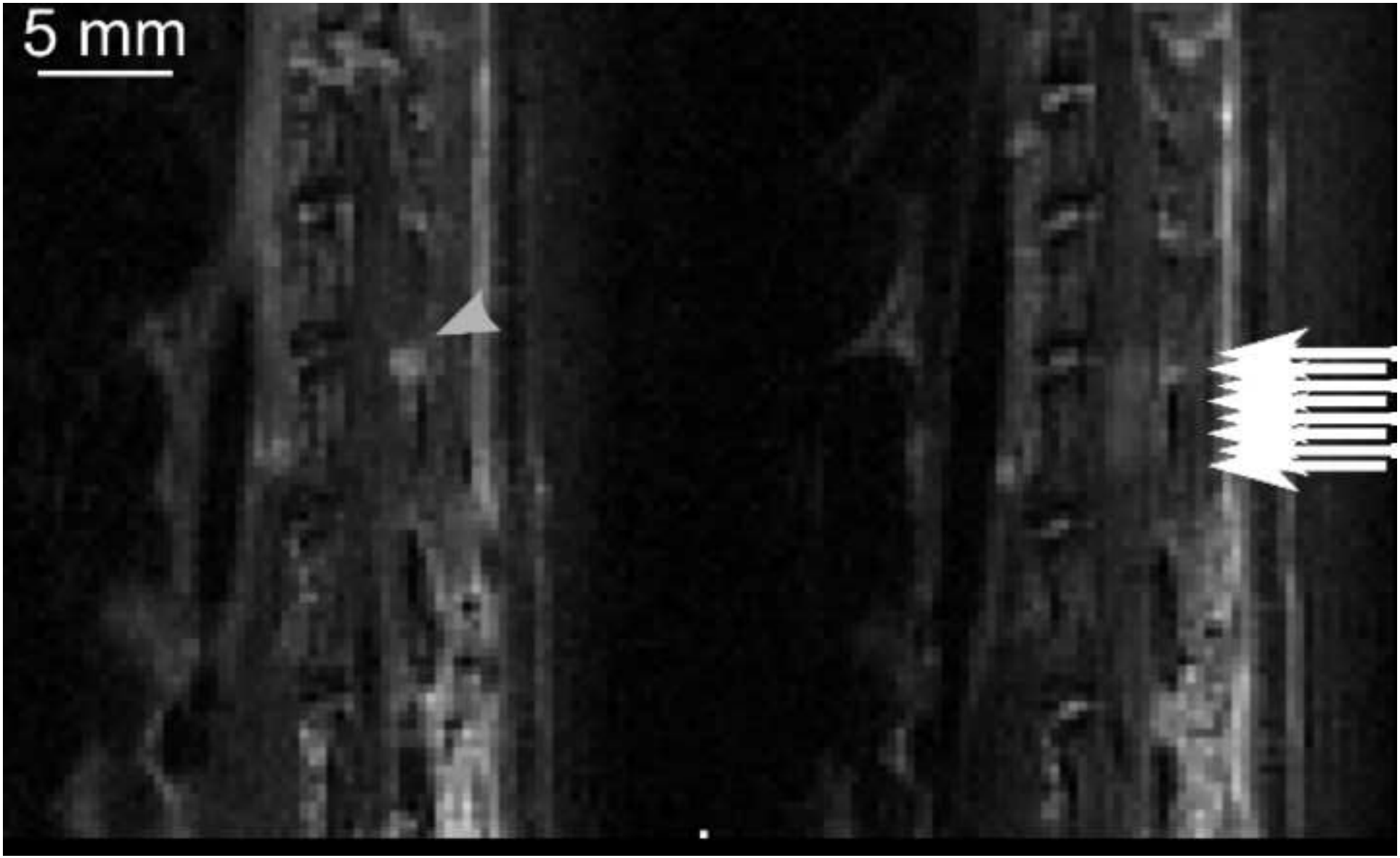


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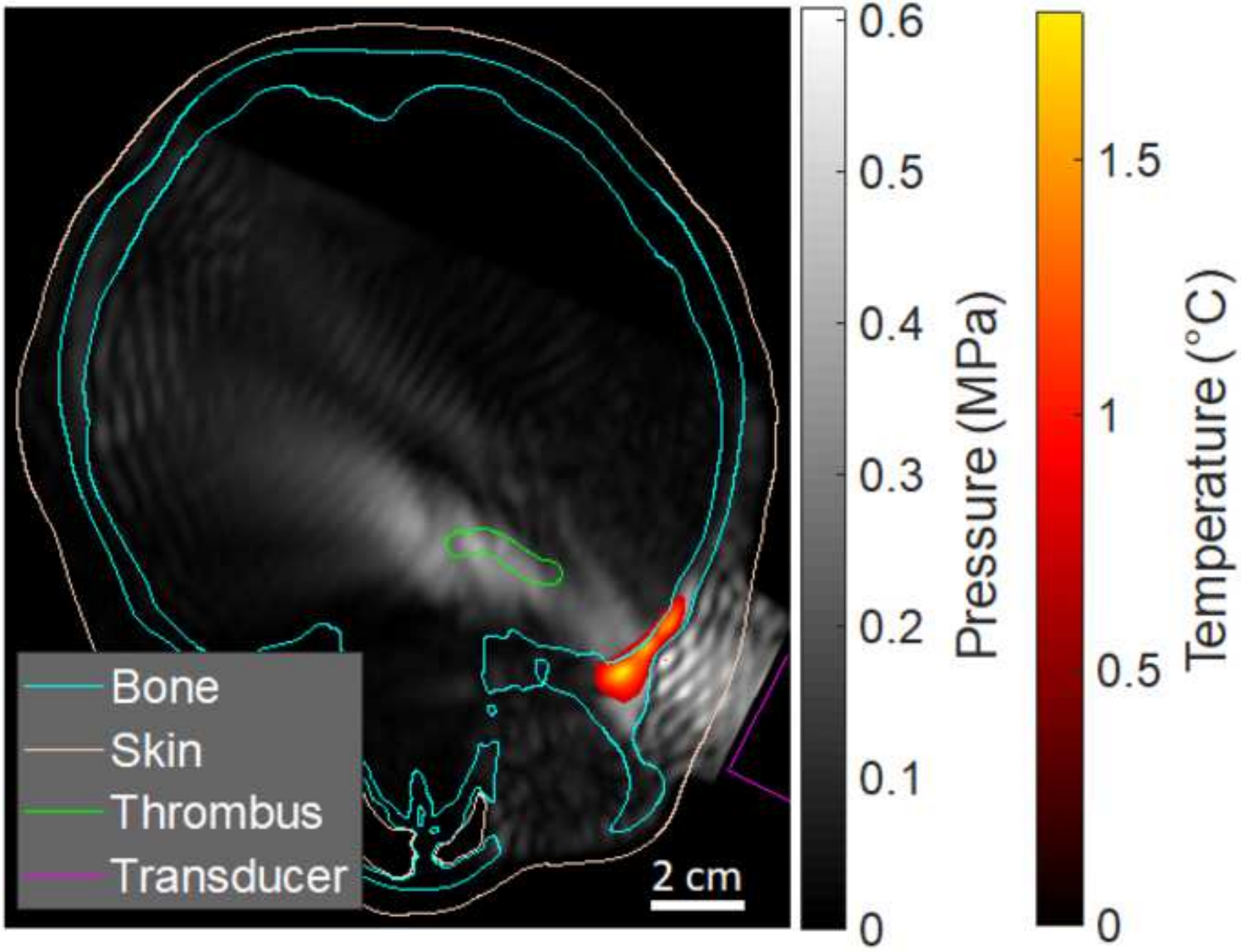


Figure 7

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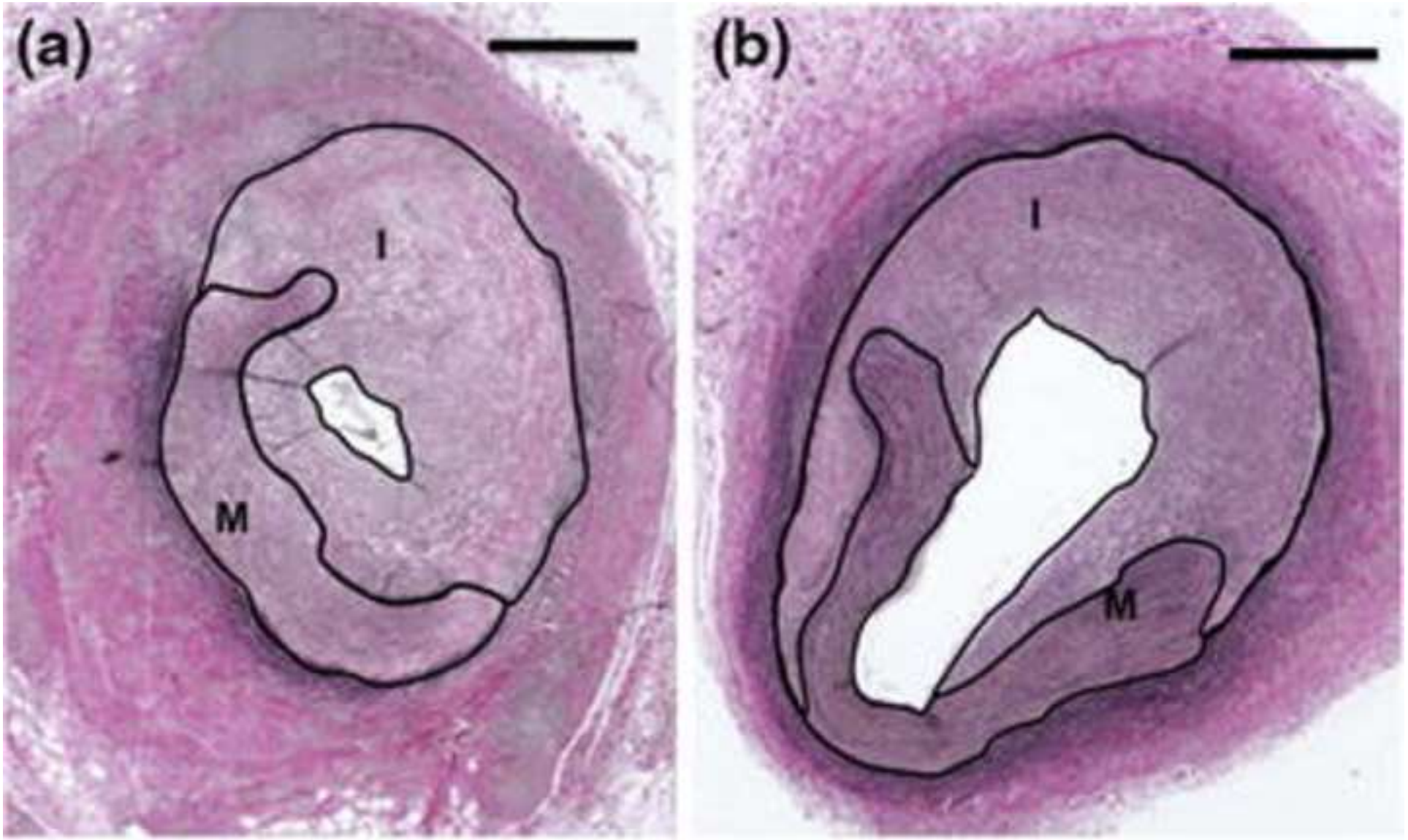


Figure 8

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