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► **To cite this version:**

Klazina Kooiman, Silke Roovers, Simone a G Langeveld, Robert T Kleven, Heleen Dewitte, et al..
Ultrasound-responsive cavitation nuclei for therapy and drug delivery. *Ultrasound in Medicine & Biology*, In press. inserm-02437994

HAL Id: inserm-02437994

<https://inserm.hal.science/inserm-02437994v1>

Submitted on 14 Jan 2020

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1 **Ultrasound-responsive cavitation nuclei for therapy and drug delivery**

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36 **ABSTRACT**

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

51

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

54 INTRODUCTION

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

74

75 CAVITATION NUCLEI FOR THERAPY

76 The most widely used cavitation nuclei are phospholipid-coated microbubbles with a gas
77 core. For the 128 preclinical studies included in the treatment sections of this review, the
78 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.

104 (2015), Dong et al. (2017), Goyal et al (2017), Pandit et al. (2019). These phospholipids are
105 popular because they are also the main component in Definity[®] (Definity[®] 2011) and
106 SonoVue[®]/Lumason[®] (Lumason[®] 2016), respectively. Another key component of the
107 microbubble coating is a PEGylated emulsifier such as polyoxyethylene (40) stearate (PEG40-
108 stearate; for example Kilroy et al. (2014)) or the most often used 1,2-distearoyl-sn-glycero-3-
109 phosphoethanolamine-N-carboxy (poly-ethyleneglycol) (DSPE-PEG2000; for example Belcik
110 et al. (2017)), which is added to inhibit coalescence and to increase the *in vivo* half-life (Ferrara,
111 et al. 2009). In general two methods are used to produce custom-made microbubbles:
112 mechanical agitation (for example Ho et al. (2018)) or probe sonication (for example Belcik et
113 al. (2015)). Both these methods produce a population of microbubbles that is polydisperse in
114 size. Monodispersed microbubbles produced by microfluidics have recently been developed,
115 and are starting to gain attention for pre-clinical therapeutic studies. Dixon et al. (2019) used
116 monodisperse microbubbles to treat ischemic stroke.

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

129 with genetic material loaded on the microbubble surface by charge-coupling (for example Cao
130 et al. (2015)). Besides phospholipids and nanoparticles, microbubbles can also be coated with
131 denatured proteins such as albumin. OptisonTM (OptisonTM 2012) is a commercially available
132 and clinically approved ultrasound contrast agent that is coated with human albumin and used
133 in studies on treatment of non-brain tumors (Xiao, et al. 2019), BBB opening (Kovacs, et al.
134 2017b, Payne, et al. 2017), and immunotherapy (Maria, et al. 2015). Nano-sized particles cited
135 in this review have been used as cavitation nuclei for treatment of tumors, such as nanodroplets
136 (for example Cao et al. (2018)) and nanocups (Myers, et al. 2016), for BBB opening
137 (nanodroplets, Wu et al. (2018)), and for sonobactericide (nanodroplets, Guo et al. (2017a)).

138

139 **BUBBLE-CELL INTERACTION**

140 **Physics**

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

143 *Physics of Microbubble – Cell Interaction*

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

151 a. Mechanical effects

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

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

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

161

$$162 \quad \ddot{R} = -\left(\frac{3}{2}\frac{\dot{R}^2}{R}\right) + \left(\frac{p_G(R)+p_\infty(t)-\frac{2\sigma}{R}}{\rho_L R}\right) = IF + PF, \quad (\text{Eq. 1})$$

163

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

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

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

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

178 deforming fragile structures such as a biological cell membranes (van Wamel, et al. 2006, Kudo
179 2017) or blood vessel walls (Chen, et al. 2011).

180 (ii) Ballistic motion – in addition to oscillating, the bubble may undergo translation as a
181 result of the pressure gradient in the fluid generated by a propagating ultrasound wave (primary
182 radiation force). Due to their high compressibility, bubbles may travel at significant velocities,
183 sufficient to push them toward targets for improved local deposition of a drug (Dayton, et al.
184 1999) or penetrate biological tissue (Caskey, et al. 2009, Bader, et al. 2015, Acconcia, et al.
185 2016).

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

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

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

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

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

217 b. Thermal effects

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

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

231 c. Chemical effects

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

240

241 *Physics of Droplets – Cell Interaction*

242 Droplets consist of an encapsulated quantity of a volatile liquid, such as perfluorobutane
243 (boiling point $-1.7\text{ }^{\circ}\text{C}$) or perfluoropentane (boiling point $29\text{ }^{\circ}\text{C}$), which is in a superheated
244 state at body temperature. Superheated state means that although the volatile liquids have a
245 boiling point below $37\text{ }^{\circ}\text{C}$, these droplets remain in the liquid phase and do not show
246 spontaneous vaporization after injection. Vaporization can be achieved instead by exposure to
247 ultrasound of significant amplitude via a process known as acoustic droplet vaporization
248 (ADV) (Kripfgans, et al. 2000). Before vaporization, the droplets are typically one order of
249 magnitude smaller than the emerging bubbles, and the perfluorocarbon is inert and
250 biocompatible (Biro and Blais 1987). These properties enable a range of therapeutic
251 possibilities (Sheeran and Dayton 2012, Lea-Banks, et al. 2019). For example, unlike
252 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, Kopeček, 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

278 larger than the applied wavelength. In the current case, the frequency of a typical ultrasound
279 wave used for insonification is in the order of 1-5 MHz, yielding wavelengths in the order of
280 1500 – 300 μm , while a droplet will be smaller by 2-4 orders of magnitude. Beside this, using
281 the ray approach, the lower speed of sound in perfluorocarbon would yield a focal spot near
282 the backside of the droplet, which is in contradiction to observations. The correct way to treat
283 the focusing effect is to solve the full diffraction problem by decomposing the incident wave,
284 the wave reflected by the droplet, and the wave transmitted into the droplet into a series of
285 spherical waves. For each spherical wave, the spherical reflection and transmission coefficients
286 can be derived. Superposition of all the spherical waves yields the pressure inside the droplet.
287 Nevertheless, when this approach is only applied to an incident wave with the frequency that
288 is emitted by the transducer, this will lead neither to the right nucleation spot nor to sufficient
289 negative pressure for vaporization. Nanoscale droplets may be too small to make effective use
290 of the focusing mechanism and ADV is therefore less dependent on the frequency.

291

292 b. Nonlinear ultrasound propagation

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

$$299 \quad p(t) = \sum_{n=1}^{\infty} a_n \cos(n\omega t + \phi_n), \quad (\text{Eq. 2})$$

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

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

312 After vaporization has started, the growth of the emerging bubble is limited by inertia and
313 heat transfer. In the absence of the heat transfer limitation, the inertia of the fluid that surrounds
314 the bubble limits the rate of bubble growth, which is linearly proportional to time and inversely
315 proportional to the square root of the density of the surrounding fluid. When inertia is
316 neglected, thermal diffusion is the limiting factor in the transport of heat to drive the
317 endothermic vaporization process of perfluorocarbon, causing the radius of the bubble to
318 increase with the square root of time. In reality, both processes occur simultaneously, where
319 the inertia effect is dominant at the early stage and the diffusion effect is dominant at the later
320 stage of bubble growth. The final size that is reached by a bubble depends on the time that a
321 bubble can expand, i.e. on the duration of the negative cycle of the insonifying pressure wave.
322 It is therefore expected that lower insonification frequencies give rise to larger maximum
323 bubble size. Thus, irrespective of their influence on triggering ADV, lower frequencies would
324 lead to more violent inertial cavitation effects and cause more biological damage, as
325 experimentally observed for droplets with a radius in the order of 100 nm (Burgess and Porter
326 2019).

327

328 **Biological mechanisms and bioeffects of ultrasound-activated cavitation nuclei**

329 The biological phenomena of sonoporation (*i.e.* membrane pore formation), stimulated
330 endocytosis, and opening of cell-cell contacts and the bioeffects of intracellular calcium
331 transients, reactive oxygen species generation, cell membrane potential change, and
332 cytoskeleton changes have been observed for several years (Sutton, et al. 2013, Kooiman, et
333 al. 2014, Lentacker, et al. 2014, Qin, et al. 2018b). However, other bioeffects induced by
334 ultrasound-activated cavitation nuclei have recently been discovered. These include membrane
335 blebbing as a recovery mechanism for reversible sonoporation (both for ultrasound-activated
336 microbubbles (Leow, et al. 2015) and upon ADV (Qin, et al. 2018a)), extracellular vesicle
337 formation (Yuana, et al. 2017), suppression of efflux transporters P-glycoprotein (Cho, et al.
338 2016, Aryal, et al. 2017) and BBB (Blood Brain Barrier) transporter genes (McMahon, et al.
339 2018). At the same time, more insight has been gained in the origin of the bioeffects, largely
340 through the use of live cell microscopy. For sonoporation, real time membrane pore opening
341 and closure dynamics were revealed with pores $<30 \mu\text{m}^2$ closing within 1 min, while pores
342 $>100 \mu\text{m}^2$ did not reseal (Hu, et al. 2013) as well as immediate rupture of filamentary actin at
343 the pore location (Chen, et al. 2014) and correlation of intracellular reactive oxygen species
344 levels with the degree of sonoporation (Jia, et al. 2018). Real-time sonoporation and opening
345 of cell-cell contacts in the same endothelial cells has been demonstrated as well for a single
346 example (Helfield, et al. 2016). The applied acoustic pressure was shown to determine uptake
347 of model drugs via sonoporation or endocytosis in another study (De Cock, et al. 2015).
348 Electron microscopy revealed formation of transient membrane disruptions and permanent
349 membrane structures, *i.e.* caveolar endocytic vesicles, upon ultrasound and microbubble-
350 treatment (Zeghimi, et al. 2015). A study by Fekri et al. (2016) revealed that enhanced clathrin-
351 mediated endocytosis and fluid-phase endocytosis occur through distinct signaling
352 mechanisms upon ultrasound and microbubble treatment. The majority of these bioeffects have

353 been observed in *in vitro* models using largely non-endothelial cells and may therefore not be
354 directly relevant to *in vivo* tissue, where intravascular micron-sized cavitation nuclei will only
355 have contact with endothelial cells and circulating blood cells. On the other hand, the
356 mechanistic studies by Belcik et al. (2015, 2017) and Yu et al. (2017) do show translation from
357 *in vitro* to *in vivo*. In these studies, ultrasound-activated microbubbles were shown to induce a
358 shear-dependent increase in intravascular adenosine triphosphate (ATP) from both endothelial
359 cells and erythrocytes, an increase in intramuscular nitric oxide, and downstream signaling
360 through both nitric oxide and prostaglandins which resulted in augmentation of muscle blood
361 flow. Ultrasound settings were similar, namely 1.3 MHz, MI 1.3 for Belcik et al. (2015, 2017)
362 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
363 negative pressure of the ultrasound wave (in MPa) and f the center frequency of the ultrasound
364 wave (in MHz).

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

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

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

383

384 **Modelling Microbubble – cell – drug interaction**

385 Whilst there have been significant efforts to model the dynamics of ultrasound driven
386 microbubbles (Faez, et al. 2013, Dollet, et al. 2019), less attention has been paid to the
387 interactions between microbubbles and cells or their impact upon drug transport. Currently
388 there are no models that describe the interactions between microbubbles, cells, and drug
389 molecules. Several models have been proposed for the microbubble – cell interaction in
390 sonoporation focusing on different aspects: the cell expansion and microbubble jet velocity
391 (Guo, et al. 2017b), the shear stress exerted on the cell membrane (Wu 2002, Doinikov and
392 Bouakaz 2010, Forbes and O'Brien 2012, Yu and Chen 2014, Cowley and McGinty 2019),
393 microstreaming (Yu and Chen 2014), shear stress exerted on the cell membrane in combination
394 with microstreaming (Li, et al. 2014), or other flow phenomena (Yu, et al. 2015, Rowlatt and
395 Lind 2017) generated by an oscillating microbubble. In contrast to the other models, Man et al.
396 (2019) propose that the microbubble-generated shear stress does not induce pore formation,
397 but that this is instead due to microbubble fusion with the membrane and subsequent “pull out”
398 of cell membrane lipid molecules by the oscillating microbubble. Models for pore formation
399 (for example Koshiyama and Wada (2011)) and resealing (Zhang, et al. 2019) in cell
400 membranes have also been developed, but these models neglect the mechanism by which the
401 pore is created. There is just one sonoporation dynamics model, developed by Fan *et al.* (2012),
402 that relates the uptake of the model drug propidium iodide (PI) to the size of the created

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

$$405 \quad F(t) = \alpha \cdot \pi D C_0 \cdot r_0 \cdot \frac{1}{\beta} (1 - e^{-\beta t}), \quad (\text{Eq. 3})$$

406 where α is the coefficient that relates the amount of PI molecules to the fluorescence intensity
407 of PI-DNA and PI-RNA, D is the diffusion coefficient of PI, C_0 is the extracellular PI
408 concentration, r_0 is the initial radius of the pore, β is the pore resealing coefficient, and t is
409 time. The coefficient α is determined by the sensitivity of the fluorescence imaging system,
410 and if unknown the equation can still be used because it is the pore size coefficient, $\alpha \cdot \pi D C_0 \cdot r_0$,
411 that determines the initial slope of the PI uptake pattern and is the scaling factor for the
412 exponential increase. A cell with a large pore will have a steep initial slope of PI uptake and
413 the maximum PI intensity quickly reaches the plateau value. A limitation of this model is that
414 equation 3 is based on two-dimensional free diffusion models, which holds for PI-RNA but not
415 for PI-DNA because this is confined to the nucleus. The model is independent of cell type, as
416 Fan et al. have demonstrated agreement with experimental results in both kidney (Fan, et al.
417 2012) and endothelial cells (Fan, et al. 2013). Other researchers have also used this model for
418 endothelial cell studies and also classified the distribution of both the pore size and pore
419 resealing coefficients using Principal Component Analysis to determine whether cells were
420 reversibly or irreversibly sonoporated. In the context of blood brain barrier (BBB) opening,
421 Hosseinkhah et al. (2015) have modeled the microbubble-generated shear and circumferential
422 wall stress for 5 μm microvessels upon microbubble oscillation at a fixed mechanical index
423 (MI) of 0.134 for a range of frequencies (0.5, 1, and 1.5 MHz). The wall stresses were
424 dependent upon microbubble size (range investigated 2 – 18 μm in diameter) and ultrasound
425 frequency. Wiedemair et al. (2017) have also modelled the wall shear stress generated by
426 microbubble (2 μm diameter) destruction at 3 MHz for larger microvessels (200 μm diameter).
427 The presence of red blood cells was included in the model and was found to cause confinement

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

431 .

432

433 **TREATMENT OF TUMORS (NON-BRAIN)**

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

442 Notwithstanding the growing knowledge of the pathophysiology of tumors, treatment
443 remains challenging. Chemotherapeutic drugs are typically administered to abolish the rapidly-
444 dividing cancer cells. Yet, their cytotoxic effects are not limited to cancer cells, causing dose-
445 limiting off-target effects. To overcome this hurdle, chemotherapeutics are often encapsulated
446 in nano-sized carriers, i.e. nanoparticles, that are designed to specifically diffuse through the
447 large openings of tumor vasculature, while being excluded from healthy tissue by normal blood
448 vessels (Lammers, et al. 2012, Maeda 2012). Despite being highly promising in pre-clinical
449 studies, drug-containing nanoparticles have shown limited clinical success due to the vast
450 heterogeneity in tumor vasculature (Barenholz 2012, Lammers, et al. 2012, Wang, et al.
451 2015d). In addition, drug penetration into the deeper layers of the tumor can be constrained
452 due to high interstitial pressure and a dense extracellular matrix in the tumor. Furthermore,

453 acidic and hypoxic regions limit the efficacy of radiation- and chemotherapy-based treatments
454 due to biochemical effects (Mehta, et al. 2012, McEwan, et al. 2015, Fix, et al. 2018).
455 Ultrasound-triggered microbubbles are able to alter the tumor environment locally, thereby
456 improving drug delivery to tumors. These alterations are schematically represented in Figure
457 2 and include: improving vascular permeability, modifying the tumor perfusion, reducing local
458 hypoxia, and overcoming the high interstitial pressure.

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

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

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

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

500 Apart from their ability to improve the tumor uptake, microbubbles can be used as
501 ultrasound-responsive drug carriers to reduce the off-target effects of chemotherapeutics. By
502 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

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

550

551 **Clinical studies**

552 *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 (Kotopoulos, et al. 2013,
556 Dimceovski, et al. 2016). In this clinical trial, gemcitabine was administered 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,

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

585

586 *Hepatic metastases from digestive system*

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

603 In addition, the severity of side effects did not worsen with increases in MI, suggesting that
604 microbubble-mediated chemotherapy is a safe procedure. The secondary aims were to assess
605 the efficacy of this therapeutic protocol using contrast-enhanced CT and MRI. Thus, tumor
606 size and development were characterized according to the RECIST criteria. Half of the patients
607 had stable disease and one patient obtained a partial response after the first treatment cycle.
608 The median progression-free survival was 91 days. However, making any comparison and
609 interpretation of results is very difficult because none of the patients were treated with the same
610 chemotherapeutics, MI, and/or number of treatment cycles. The results of safety and efficacy
611 evaluations should be compared to patients receiving the chemotherapy on its own in order to
612 clearly identify the therapeutic benefit of combining with ultrasound-driven microbubbles.
613 Similar to the pancreatic clinical study, no direct evidence of enhanced therapeutic
614 bioavailability of the chemotherapeutic drug after the treatment was provided. This
615 investigation is all the more important as the ultrasound and microbubble treatment was applied
616 30 min after intravenous chemotherapy (for both monotherapy and combination therapy)
617 independently of drug pharmacokinetics and metabolism.

618

619 *Ongoing and upcoming clinical trials*

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

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

641

642 **IMMUNOTHERAPY**

643 Cancer immunotherapy is considered to be one of the most promising strategies to eradicate
644 cancer as it makes use of the patient's own immune system to selectively attack and destroy
645 tumor cells. It is a common name that refers to a variety of strategies that aim to unleash the
646 power of the immune system by either boosting antitumoral immune responses or flagging
647 tumor cells to make them more visible to the immune system. The principle is based on the
648 fact that tumors express specific tumor antigens which are not, or to a much lesser extent,
649 expressed by normal somatic cells and hence can be used to initiate a cancer-specific immune
650 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

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

691 Another strategy in cancer immunotherapy is adoptive cell therapy, where *ex vivo*
692 manipulated immune effector cells, mainly T cells and NK (natural killer) cells, are employed
693 to generate a robust and selective anticancer immune response (Yee 2018, Hu, et al. 2019).
694 These strategies have mainly led to successes in hematological malignancies, not only because
695 of the availability of selective target antigens, but also because of the accessibility of the
696 malignant cells (Khalil, et al. 2016, Yee 2018). By contrast, in solid tumors, and especially in
697 brain cancers, inadequate homing of cytotoxic T cells or NK cells to the tumor proved to be
698 one of the main reasons for the low success rates, making the degree of tumor infiltration an
699 important factor in disease prognosis (Childs and Carlsten 2015, Gras Navarro, et al. 2015, Yee
700 2018). To address this, focused ultrasound and microbubbles have been used to make tumors

701 more accessible to cellular therapies. The first demonstration of this concept was provided by
702 Alkins et al. (2013) who used a xenograft HER-2-expressing breast cancer brain metastasis
703 model to determine whether ultrasound and microbubbles could allow intravenously infused
704 NK cells to cross the blood-brain barrier (BBB). By loading the NK cells with
705 superparamagnetic iron oxide (SPIO) nanoparticles, the accumulation of NK cells in the brain
706 could be tracked and quantified via MRI. An enhanced accumulation of NK cells was found
707 when the cells were injected immediately prior to BBB disruption. Importantly NK cells
708 retained their activity and ultrasound treatment resulted in a sufficient NK to tumor cell ratio
709 to allow effective tumor cell killing (Alkins, et al. 2016). In contrast, very few NK cells reached
710 the tumor site when BBB disruption was absent or performed before NK cell infusion.
711 Although it is not known for certain why timing had such a significant impact on NK
712 extravasation, it is likely that the most effective transfer to the tissue occurs at the time of
713 insonification, and that the barrier is most open during this time (Marty, et al. 2012). Possible
714 other explanations include the difference in size of the temporal BBB openings or a possible
715 alternation in the expression of specific leukocyte adhesion molecules by the BBB disruption,
716 thus facilitating the translocation of NK cells. Also for tumors where BBB crossing is not an
717 issue, ultrasound has been used to improve delivery of cellular therapeutics. Sta Maria et al.
718 (2015) demonstrated enhanced tumor infiltration of adoptively transferred NK cells after
719 treatment with microbubbles and low dose focused ultrasound. This result was confirmed by
720 Yang *et al.* (2019a) in a more recent publication where the homing of NK cells was more than
721 doubled after microbubble injection and ultrasound treatment of an ovarian tumor. Despite the
722 enhanced accumulation, however, the authors did not observe an improved therapeutic effect,
723 which might be due to the limited number of treatments that were applied, or the
724 immunosuppressive tumor microenvironment that counteracts the cytotoxic action of the NK
725 cells.

726 There is growing interest in exploring the effect of microbubbles and ultrasound on the
727 tumor microenvironment, as recent work has shown that BBB disruption with microbubbles
728 and ultrasound may induce sterile inflammation. Although a strong inflammatory response may
729 be detrimental in the case of drug delivery across the BBB, it might be interesting to further
730 study this inflammatory response in solid tumors as it might induce the release of damage-
731 associated molecular patterns (DAMPS) such as heat-shock proteins and inflammatory
732 cytokines. This could shift the balance towards a more inflammatory microenvironment that
733 could promote immunotherapeutic approaches. As reported by Liu *et al.* (2012) exposure of a
734 CT26 colon carcinoma xenograft to microbubbles and low pressure pulsed ultrasound
735 increased cytokine release and triggered lymphocyte infiltration. Similar data have been
736 reported by Hunt *et al.* (2015). In their study, ultrasound treatment caused a complete shut-
737 down of tumor vasculature followed by the expression of HIF-1 α (hypoxia-inducible factor
738 1 α), a marker of tumor ischemia and tumor necrosis, as well as increased infiltration of T cells.
739 Similar responses have been reported following thermal and mechanical HIFU treatments of
740 solid tumors (Unga and Hashida 2014, Silvestrini, et al. 2017). A detailed review of ablative
741 ultrasound therapies is however out of the scope of this review.

742 At present, the most successful form of immunotherapy is the administration of monoclonal
743 antibodies to inhibit regulatory immune checkpoints that block T cell action. Examples are
744 CTLA-4 (cytotoxic T lymphocyte-associated protein-4) and PD-1 (programmed cell death-1),
745 which act as brakes on the immune system. Blocking the effect of these brakes can revive and
746 support the function of immune effector cells. Despite the numerous successes achieved with
747 checkpoint inhibitors, responses have been quite heterogeneous as the success of checkpoint
748 inhibition therapy largely depends on the presence of intratumoral effector T cells (Weber
749 2017). This motivated Bulner et al. (2019) to explore the synergy of microbubble and
750 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.

776

777 **Mechanisms, Bioeffects, and Safety**

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

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

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

801 exogenous therapeutics. These effects include a reduction in amyloid- β plaque load (Jordão, et
802 al. 2013, Burgess, et al. 2014, Leinenga and Götz 2015, Poon, et al. 2018), reduction in tau
803 pathology (Pandit, et al. 2019), and improvements in spatial memory (Burgess, et al. 2014,
804 Leinenga and Götz 2015). Two-photon microscopy has shown that amyloid- β plaque size is
805 reduced in transgenic mice for up to two weeks post ultrasound and microbubble treatment
806 (Poon, et al. 2018). Opening of the BBB in both transgenic and wild-type mice has also
807 revealed enhanced neurogenesis (Burgess, et al. 2014, Scarcelli, et al. 2014, Mooney, et al.
808 2016) in the treated tissue.

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

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

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

831

832 **Cavitation Monitoring and Control**

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

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

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

863

864 **BSCB opening**

865 Despite the similarities between the BBB and BSCB, and the great potential benefit for
866 patients, there has been limited work investigating translation of this technology to the spinal
867 cord. Opening of the BSCB in rats was first reported by Wachsmuth et al. (2009), and was
868 followed by studies from Weber-Adrien et al. (2015), Payne et al. (2017), and O'Reilly et al.
869 (2018) in rats (Figure 5) and from Montero et al. (2019) in rabbits, the latter performed through
870 a laminectomy window. In 2018, O'Reilly et al. (2018) presented the first evidence of a
871 therapeutic benefit in a disease model, showing improved tumor control in a rat model of
872 leptomeningeal metastases.

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

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

883

884 **Clinical studies**

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

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

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

904 Another study conducted in Alzheimer's disease patients with the Exablate device
905 demonstrated safe BBB permeability enhancement and that the treatment could be repeated
906 one month later without any imaging or behavior indications of adverse events (Lipsman, et al.
907 2018). A third study with the same device investigated the feasibility of using functional MRI
908 to target motor cortex in Amyotrophic Lateral Sclerosis (ALS) patients again showing precisely
909 targeted BBB permeability enhancement without adverse effects in this delicate structure
910 (Abraham, et al. 2019). All of these studies were conducted using Definity® microbubbles.
911 These studies have led to the current ongoing brain tumor trial with six monthly treatments of
912 the brain tissue surrounding the resection cavity during the maintenance phase of the treatment
913 with temozolomide. This study sponsored by InSightec is being conducted in multiple
914 institutions. Similarly, a phase II trial in Alzheimer's disease sonicating the hippocampus with
915 the goal of investigating the safety and potential benefits from repeated (three treatments with
916 two-week interval) BBB permeability enhancement alone is ongoing. This study is also being
917 conducted in several institutions that have the device.

918

919 **SONOTROMBOLYSIS**

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

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

930

931 **Mechanisms, Agents, and Approaches**

932 Ultrasound improves recombinant tissue plasminogen activator (rt-PA) diffusion into
933 thrombi and augments lysis primarily via acoustic radiation force and streaming (Datta, et al.
934 2006, Prokop, et al. 2007, Petit, et al. 2015). Additionally, ultrasound increases rt-PA and
935 plasminogen penetration into the thrombus surface and enhances removal of fibrin degradation
936 products via ultrasonic bubble activity, or acoustic cavitation, that induces microstreaming
937 (Elder 1958, Datta, et al. 2006, Sutton, et al. 2013). Two types of cavitation are correlated with
938 enhanced thrombolysis: stable cavitation, with highly nonlinear bubble motion resulting in
939 acoustic emissions at the subharmonic and ultraharmonics of the fundamental frequency (Flynn
940 1964, Phelps and Leighton 1997, Bader and Holland 2013), and inertial cavitation, with
941 substantial radial bubble growth and rapid collapse generating broadband acoustic emissions
942 (Carstensen and Flynn 1982, Flynn 1982).

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

951 shelled microbubbles (Weller, et al. 2002) as a nucleation agent. However, Kleven et al. (2019)
952 observed more than 60% fractional clot width loss for highly retracted human whole blood
953 clots exposed to rt-PA, Definity[®] and 220 kHz pulsed or continuous wave (CW) ultrasound at
954 an acoustic output with sustained stable cavitation throughout the insonification periods
955 (0.22 MPa peak rarefactional pressure) (Figure 6).

956 Echogenic liposomes loaded with rt-PA enhanced lysis compared to rt-PA alone at
957 concentrations of 1.58 and 3.15 mg/mL (Shekhar, et al. 2017), suggesting that encapsulation
958 of rt-PA could reduce the rt-PA dose by a factor of two with equivalent lytic activity.
959 Subsequently it has been demonstrated that these liposomes protect rt-PA against degradation
960 by plasminogen activator inhibitor-1 (PAI-1), while achieving equivalent thrombolytic
961 efficacy relative to rt-PA, Definity[®], and intermittent 220 kHz CW ultrasound (Shekhar, et al.
962 2019). Promising agents, including a nanoscale (< 100 nm) contrast agent (Brüssler, et al.
963 2018) and magnetically targeted microbubbles (De Saint Victor, et al. 2019), have also
964 demonstrated enhanced rt-PA thrombolysis *in vitro*. All of these investigators noted that in the
965 absence of rt-PA, the combination of ultrasound and microbubbles did not degrade the fibrin
966 network.

967 Several minimally invasive techniques have also been explored, with or without the
968 inclusion of rt-PA or exogenous cavitation nuclei. In the clinical management of stroke, rapid
969 treatments are needed because of the neurologist's adage "time is brain". Thus, treatment
970 options that promote fast clot removal, reduce edema and intracerebral bleeding, and improve
971 patient outcomes are of immense value. Magnetic resonance image-guided high intensity
972 focused ultrasound has been investigated for the treatment of both ischemic (Burgess, et al.
973 2012) and hemorrhagic (Monteith, et al. 2013) stroke, and Zafar et al. (2019) have provided an
974 excellent review of the literature for this approach. Histotripsy, a form of high intensity focused
975 ultrasound that relies on the mechanical action of microbubble clouds to ablate thrombi with

976 and without rt-PA (Maxwell, et al. 2009, Bader, et al. 2015, Zhang, et al. 2016b, Bader, et al.
977 2019) is under development to treat deep vein thrombosis. Additionally, ultrasound-accelerated
978 catheter-directed thrombolysis using the EKOS system (EKOS/BTG, Bothell, WA, USA)
979 combines 2 MHz low-intensity pulsed ultrasound and rt-PA without cavitation nuclei to
980 improve lytic efficiency to treat deep vein thrombosis (Shi, et al. 2018) and pulmonary
981 embolism (Garcia 2015).

982

983 **Cavitation monitoring**

984 Acoustic cavitation has been shown to mediate direct fibrinolysis (Weiss, et al. 2013) and
985 accelerated rt-PA lysis (Everbach and Francis 2000, Datta, et al. 2006, Prokop, et al. 2007,
986 Hitchcock, et al. 2011). Passive and active cavitation detection techniques have been developed
987 to monitor acoustic cavitation (Roy, et al. 1990, Madanshetty, et al. 1991, Bader, et al. 2015).
988 Passive cavitation imaging, or passive acoustic mapping, employs a transducer array that
989 listens passively (i.e., no transmit) to emissions from acoustically activated microbubbles
990 (Salgaonkar, et al. 2009, Gyöngy and Coussios 2010, Haworth, et al. 2017). Vignon et al.
991 (2013) developed a prototype array enabling spectral analysis of bubble activity for
992 sonothrombolysis applications. Superharmonic Doppler effects have also been utilized to
993 monitor bubble activity from 500 kHz pulsed therapeutic ultrasound (Pouliopoulos and Choi
994 2016). Both a linear array (Arvanitis and McDannold 2013a, Arvanitis, et al. 2013, Arvanitis
995 and McDannold 2013b) and a sparse hemispherical array (Acconcia, et al. 2017) have been
996 integrated into a clinical magnetic resonance image-guided high intensity focused ultrasound
997 system to assess microbubble dynamics during sonothrombolysis in the brain.

998

999 **Preclinical studies**

1000 Information gathered from animal studies can help inform human clinical trials, despite a
1001 strong species dependence of clot rt-PA lytic susceptibility (Gabriel, et al. 1992, Flight, et al.
1002 2006, Huang, et al. 2017). A comprehensive systematic evaluation of 16 *in vivo* preclinical
1003 sonothrombolysis studies was carried out by Auboire et al. (2018) summarizing treatment
1004 efficacy and safety outcomes in models of ischemic stroke. Since that review was published,
1005 the efficacy of sonothrombolysis using nitrogen microbubbles stabilized with a non-
1006 crosslinked shell delivered intra-arterially through a catheter and rt-PA delivered intravenously
1007 has been demonstrated in a rat model of ischemic stroke (Dixon, et al. 2019).

1008

1009 **Clinical studies**

1010 A rich literature exists of clinical trials exploring the safety and efficacy of
1011 sonothrombolysis. Two recent meta-analyses of seven randomized controlled trials (Chen, et
1012 al. 2019, Zafar, et al. 2019) attempt to determine whether the administration of rt-PA and
1013 ultrasound improve outcomes in acute ischemic stroke. Both analyses conclude that
1014 sonothrombolysis significantly enhances complete or partial recanalization, with improved
1015 neurologic function (assessed via the National Institutes of Health Stroke Scale, NIHSS). An
1016 ongoing clinical trial (TRUST; NCT03519737) will determine whether large vessel occlusions
1017 can be recanalized with sonothrombolysis (Cerevast Medical, Inc., Bothell, WA, USA) and rt-
1018 PA, tenecteplase or alteplase, (Campbell, et al. 2018) while patients are transferred to a stroke
1019 center for mechanical thrombectomy (Gauberti 2019).

1020 Several clinical trials have shown that high MI pulsed diagnostic ultrasound exposure of
1021 Definity® before and after percutaneous coronary intervention for ST elevation myocardial
1022 infarction can prevent microvascular obstruction and improve functional outcomes (Mathias,
1023 et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019). A systematic review of 16 catheter-
1024 directed sonothrombolysis clinical trials comprised mostly of retrospective case series using

1025 the EKOS system without microbubble infusions determined that this treatment modality is
1026 safe and promising for the treatment of deep vein thrombosis, DVT (Shi, et al. 2018). However,
1027 a large-sample randomized prospective clinical trial is needed to improve the clinical evidence
1028 for use as a front-line therapy for DVT. In retrospective studies in patients with pulmonary
1029 embolism Lee et al. (2017) conclude that catheter directed sonothrombolysis is safe and
1030 decreases right-sided heart strain, but Schissler et al. (2018) conclude that this therapy is not
1031 associated with a reduction in mortality nor increased resolution of right ventricular
1032 dysfunction. And finally, an ongoing trial in a small cohort of 20 patients with acute peripheral
1033 arterial occlusions (Ebben, et al. 2017) will determine whether Luminity[®] (marketed in the US
1034 as Definity[®]) and 1.8 MHz transdermal diagnostic ultrasound with intermittent high MI (1.08)
1035 and low MI (0.11) for visualization of the microbubbles and flow will improve recanalization.
1036 In summary, sonothrombolysis has demonstrated clinical benefit in the treatment of acute and
1037 chronic thrombotic disease. Ultrasound-assisted thrombolysis has a potential role as an
1038 emerging viable and therapeutic option for future management of stroke and cardiovascular
1039 disease.

1040

1041 **CARDIOVASCULAR DRUG DELIVERY AND THERAPY**

1042 In cardiovascular drug delivery, cavitation nuclei are co-administered or loaded with
1043 different therapeutics for the treatment of various diseases. For atherosclerosis treatment in an
1044 ApoE-deficient mouse model, intercellular adhesion molecule-1 targeted microbubbles
1045 carrying angiogenesis inhibitor Endostar were used (Yuan, et al. 2018). Upon intermittent
1046 insonification over the abdominal and thoracic cavity with 1 MHz ultrasound (2 W/cm²
1047 intensity, 50% duty cycle) for 30 s with two repeats and another treatment 48 h later, plaque
1048 area and intraplaque neovascularization were significantly reduced two weeks after treatment.
1049 Percutaneous coronary intervention is often used to restore blood flow in atherosclerotic

1050 arteries. The treatment of coronary microembolization, a complication of percutaneous
1051 coronary intervention, was demonstrated in pigs treated with ultrasound (1 MHz, 2.0 W/cm²
1052 intensity, 10 s on and 10 s off, 20 min duration) and microRNA-21-loaded microbubbles four
1053 days before coronary microembolization (Su, et al. 2015). This resulted in an improved cardiac
1054 dysfunction. Although not a therapeutic study, Liu et al. (2015) did show that plasmid
1055 transfection to the myocardium was significantly larger when the microbubbles were
1056 administered into the coronary artery compared to intravenously via the ear vein in pigs even
1057 though the intracoronary microbubble dose was half of the intravenous dose (1 MHz
1058 ultrasound, 2 W/cm², 50% duty cycle, 20 min duration). Percutaneous coronary intervention
1059 can also result in neointimal formation which induces restenosis. Sirolimus-loaded
1060 microbubbles were shown to reduce neointimal formation in coronary arteries by 50% in pigs,
1061 see Figure 7, 28 days after angioplasty in combination with a mechanically rotating
1062 intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative
1063 pressure) (Kilroy, et al. 2015). Another research group showed that paclitaxel-loaded
1064 microbubbles and ultrasound (1 MHz, 1.5 MPa for 10 s) can also significantly inhibit
1065 neointimal formation in the iliac artery in rabbits one week after percutaneous coronary
1066 intervention (Zhu, et al. 2016).

1067 In diabetic cardiomyopathy, microbubble-mediated delivery of fibroblast growth factor has
1068 shown therapeutic effects. Zhao et al (2016) could prevent diabetic cardiomyopathy in rats by
1069 treating the heart with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of
1070 1 s) and microbubbles co-administered with acidic fibroblast growth factor nanoparticles twice
1071 weekly for 12 consecutive weeks. In already established diabetic cardiomyopathy in rats, the
1072 same investigators co-administered basic fibroblast growth factor-containing nanoparticles
1073 with microbubbles with the same ultrasound treatment, albeit that it was given three times with
1074 one day in between treatments. At four weeks after treatment, this resulted in restored cardiac

1075 functions as a result of structural remodeling of the cardiac tissue (Zhao, et al. 2014).
1076 Microbubbles loaded with acidic fibroblast growth factor in combination with ultrasound (14
1077 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) also showed significantly
1078 improved cardiac function in a rat model of diabetic cardiomyopathy. Treatment was
1079 performed twice weekly for 12 consecutive weeks (Zhang, et al. 2016a). For doxorubicin
1080 induced cardiomyopathy, repeated co-administration of microbubbles and nanoparticles
1081 containing acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa
1082 for 10 s, three repeats with off interval of 1 s) applied at the heart successfully prevented
1083 doxorubicin induced cardiomyopathy in rats (Tian, et al. 2017). Once doxorubicin induced
1084 cardiomyopathy had occurred, microbubble-mediated reversal of cardiomyopathy was shown
1085 by the delivery of survivin plasmid to cardiomyocytes and endothelial cells (Lee, et al. 2014)
1086 or glucagon-like peptide-1 (GLP-1) to cardiomyocytes, endothelial cells, vascular muscle cells,
1087 and mesenchymal cells (Chen, et al. 2015) in rats. The ultrasound settings were 5 MHz (120 V
1088 power, pulsing interval of 10 cardiac cycles at end-systole) for a 5 min treatment (Lee, et al.
1089 2014) or not specified (Chen, et al. 2015). The microbubble-mediated gene therapy study by
1090 Chen et al. (2016) showed that ANGPTL8 gene therapy does not need to be done in the heart
1091 to reverse doxorubicin induced cardiomyopathy in rats as their microbubble and ultrasound
1092 (1.3 MHz, 1.4 MPa peak negative pressure, four bursts triggered to every fourth end-systole
1093 using a delay of 45-70 ms of the peak of the R wave) therapy was done in the liver (90 s
1094 treatment). This resulted in overexpression of ANGPTL8 in liver cells and blood which
1095 stimulated cardiac progenitor cells in the epicardium.

1096 A few dozen articles have been published on treating myocardial infarction with
1097 microbubble and ultrasound-mediated gene delivery *in vivo*, in mouse, rat, rabbit, and dog
1098 models. These are reviewed by Qian et al. (2018). Amongst these are a few targeted
1099 microbubble studies which all show that the targeted microbubbles induced higher degrees of

1100 gene transfection, increased myocardial vascular density, and improved cardiac function in
1101 comparison to non-targeted microbubbles. This improvement occurred independent of the type
1102 of ligand on the microbubble, the gene that was transfected, or the animal model: matrix
1103 metalloproteinase 2 target with Timp3 gene in rats (Yan, et al. 2014), intracellular adhesion
1104 molecule-1 target with Ang-1 gene in rabbits (Deng, et al. 2015), P-selectin target with
1105 hVEGF165 gene in rats (Shentu, et al. 2018). Ultrasound settings for these studies were similar
1106 at 1.6 MHz (1.6 MPa peak negative pressure, pulsing interval of four cardiac cycles) for 20
1107 min during infusion of the plasmid-loaded microbubbles (both Yan et al. (2014) and Shentu et
1108 al. (2018)), or 1.7 MHz (1.7 MPa peak negative pressure, pulsing interval every four to eight
1109 cardiac cycles) for 5 min after bolus injection of the plasmid-loaded microbubbles (Deng, et
1110 al. 2015).

1111 Other gene therapy studies for vascular disease include stimulating angiogenesis for the
1112 treatment of chronic hindlimb ischemia in rats using miR-126-3p-loaded microbubbles and
1113 ultrasound (1.3 MHz, 2.1 MPa peak negative acoustic pressure, pulsing interval 5 s). The
1114 treatment lasted for 20 min of which microbubbles were infused for 10 min and resulted in
1115 improved perfusion, vessel density, arteriolar formation, and neovessel maturation (Cao, et al.
1116 2015). Recently, successful gene therapy was demonstrated in baboons where Vascular
1117 Endothelial Growth Factor (VEGF)-plasmid loaded microbubbles were infused and ultrasound
1118 (2-6 MHz, MI 1.9, repeated 5 s burst pulses with three bursts per minute) was applied for 10
1119 min on days 25, 35, 45, and 55 of gestation with the transducer placed over the placental basal
1120 plate (Babischkin, et al. 2019). This was a mechanistic study elucidating the role of VEGF in
1121 uterine artery remodeling.

1122 The gas core of the cavitation nuclei can also be the therapeutic. Sutton et al. (2014) have
1123 shown that ultrasound-mediated (1 MHz, 0.34 MPa acoustic pressure, 30 cycle pulse, 50 s
1124 treatment) nitric oxide gas delivery from echogenic liposomes to *ex vivo* perfused porcine

1125 carotid arteries induces potent vasorelaxation. The vasodialative effect of nitric oxide-loaded
1126 echogenic liposomes upon insonification (5.7 MHz, 0.36 MPa peak negative pressure, 30 s
1127 treatment) was also shown in *ex vivo* perfused rabbit carotid arteries with arterial wall
1128 penetration of nitric oxide confirmed by fluorescence microscopy (Kim, et al. 2014). In
1129 addition to this, vasodialative effects were demonstrated in carotid arteries *in vivo* in rats with
1130 vasospasms following subarachnoid hemorrhage using 1 MHz ultrasound with 0.3 MPa peak-
1131 to-peak pressure, 50% duty cycle for a duration of 40 min with constant infusion of the
1132 echogenic liposomes. This resulted in improved neurological function (limb placement, beam
1133 and grid walking) (Kim, et al. 2014). Ultrasound-activation of the antioxidant hydrogen gas
1134 encapsulated in microbubbles was shown to prevent myocardial ischemia-reperfusion injury in
1135 rats when administered before reperfusion (He, et al. 2017). There was a dose-dependent effect
1136 as 2×10^{10} microbubbles resulted in a more significant reduction in infarct size (70%) than 4
1137 $\times 10^9$ microbubbles (39%) compared to vehicle-treated rats. Furthermore, treatment with the
1138 high dose hydrogen-microbubbles prevented changes in left ventricular end-diastolic and left
1139 ventricular end-systolic dimension as well as minimal reductions in ejection fraction and
1140 fractional shortening. Histological and ELISA analysis showed a reduced degree of myocardial
1141 necrosis, apoptosis, hemorrhaging, inflammation, and oxidant damage. At the same time that
1142 cardiovascular drug delivery and therapy using microbubbles and ultrasound is moving
1143 forward to large animal and clinical studies, sophisticated *in vitro* models are being used and/or
1144 developed for mechanistic studies, such as flow chambers (μ Slides, Ibidi) (Shamout, et al.
1145 2015) and perfused 3D microvascular networks (Juang, et al. 2019) in which human umbilical
1146 vein endothelial cells are grown.

1147

1148 **Clinical study**

1149 Microbubbles and ultrasound were clinically investigated to augment muscle blood flow in
1150 12 patients with stable sickle cell disease in the absence of a drug at the Oregon Health &
1151 Science University, Portland, Oregon, USA (Belcik, et al. 2017). Perfusion increased ~2-fold
1152 in the forearm flexor muscles upon Definity[®] infusion and insonification at 1.3 MHz (MI 1.3).
1153 Ultrasound was applied 3 times for 3 min with ~5 min intervals. The change in perfusion was
1154 determined from contrast enhanced ultrasound imaging and extended well beyond the region
1155 where ultrasound was applied. This study showed that the therapeutic ultrasound settings
1156 directly translate from mouse to man for superficial muscles, as the same investigators
1157 demonstrated augmented blood flow in ischemic and non-ischemic hindlimb muscles in mice
1158 in the same study and an earlier publication (Belcik, et al. 2015). However, for the preclinical
1159 studies custom-made microbubbles were used instead of Definity[®].

1160

1161 **SONOBACTERICIDE**

1162 Sonobactericide has been defined as the use of ultrasound in the presence of cavitation
1163 nuclei for the enhancement of bactericidal action (Lattwein, et al. 2018). This topic has recently
1164 gained attention with 17 papers being published in the last five years. Research on ultrasound-
1165 mediated enhancement of antimicrobials has focused on several sources of infections including
1166 general medical devices (Ronan, et al. 2016, Dong, et al. 2017, Dong, et al. 2018, Hu, et al.
1167 2018, Fu, et al. 2019), acne (Liao, et al. 2017), chronic bacterial prostatitis (Yi, et al. 2016),
1168 infective endocarditis (Lattwein, et al. 2018), pneumonia (Sugiyama, et al. 2018), prosthetic
1169 joint infections (Li, et al. 2015, Lin, et al. 2015, Guo, et al. 2017a, Zhou, et al. 2018), or urinary
1170 tract infections (Horsley, et al. 2019). However, there was no specific disease aim in two studies
1171 (Zhu, et al. 2014, Goh, et al. 2015). One group targeted membrane biofouling for water and
1172 wastewater industries (Agarwal, et al. 2014). Direct bacterial killing, biofilm degradation and
1173 dispersal, and increased or synergistic therapeutic effectiveness of antimicrobials have been

1174 reported as the therapeutic effects of sonobactericide. These studies show that sonobactericide
1175 can be applied to treat Gram+ or Gram- bacteria, when they are planktonic, associated with a
1176 surface and embedded in biofilm, or intracellular. The majority of these studies were carried
1177 out *in vitro*. However, seven were performed *in vivo* in either mice (Li, et al. 2015, Liao, et al.
1178 2017, Sugiyama, et al. 2018, Zhou, et al. 2018), rats (Yi, et al. 2016), or rabbits (Lin, et al.
1179 2015, Dong, et al. 2018). Sonobactericide was mostly performed with co-administration of
1180 antimicrobials. Investigators also employed an antimicrobial encapsulated in liposomes that
1181 were conjugated to the microbubbles (Horsley, et al. 2019), or the antimicrobial lysozyme was
1182 a microbubble coating (Liao, et al. 2017), or did not use antimicrobials altogether (Agarwal, et
1183 al. 2014, Goh, et al. 2015, Yi, et al. 2016). An extensive review of sonobactericide has been
1184 published recently by Lattwein et al. (2019). Although sonobactericide is an emerging strategy
1185 to treat bacterial infections with intriguing potential, the mechanism and the safety of the
1186 treatment should be explored, particularly regarding biofilm degradation and dispersal. Future
1187 studies should also focus on maximizing the efficacy of sonobactericide *in situ*.

1188

1189 **FUTURE PERSPECTIVES AND CONCLUSIONS**

1190 Therapeutic ultrasound technology is experiencing a paradigm shift in terms of both
1191 technical developments and clinical applications. In addition to its inherent advantages for
1192 imaging (e.g., real time nature, portability and low cost), ultrasound in combination with
1193 cavitation nuclei is under exploration as a drug delivery modality. The results from several
1194 preclinical studies have already demonstrated the potential of ultrasound-responsive cavitation
1195 nuclei to deliver multiple types of drugs (including model drugs, anticancer, therapeutic
1196 antibodies, genes, nanoparticles, etc.) efficiently in various tumor models, including both
1197 ectopic and orthotopic models, for immunotherapy, brain disease, to promote the dissolution
1198 of clots, and in the treatment of cardiovascular disease and bacterial infections.

1199 Based on these encouraging preclinical data, several clinical trials have been initiated and
1200 others are planned. However, whilst animal studies provide proof of concept, and impetus for
1201 clinical studies, careful attention must be given to their relevance in human disease; in
1202 particular, the applicability of therapeutic protocols, and appropriate ultrasound settings.
1203 Otherwise we risk underestimating the therapeutic effects and potential deleterious side effects.
1204 The elucidation of all of the interactions between cavitation nuclei – cells and drugs will help
1205 to address this need. The biggest challenges lie in the large differences in timescales between
1206 the cavitation nuclei, drug release and uptake, and the biological response (Figure 8). A
1207 multidisciplinary approach is needed to tackle these challenges integrating expertise in physics,
1208 biophysics, biology, chemistry, and pharmacology.

1209 Custom-made microbubbles which serve as cavitation nuclei are often used for ultrasound-
1210 mediated drug delivery studies. An advantage is full control over the payload, as well as the
1211 disease target. At the same time, full acoustical characterization and sterility of the
1212 microbubbles must be considered during translation to human studies, which often requires
1213 approval from the United States Food and Drug Administration (FDA) or other similar federal
1214 agencies in Europe and Asia. As an example, for gene therapy, will each different type of
1215 genetic material loaded onto microbubbles need such approval, or will a class of cationic
1216 microbubbles be approved regardless of the specific gene? The former path would hinder fast
1217 clinical translation. For now, co-administration of drugs with FDA-approved ultrasound
1218 contrast agents is being explored in clinical trials. Apart from applications in the brain, ongoing
1219 clinical studies evaluating microbubble-mediated drug delivery are based on standard clinical
1220 ultrasound scanners operating mostly in Doppler mode. In order to promote the progress of this
1221 emerging technology, it is very important to design and implement specific therapeutic
1222 ultrasound pulse sequences that might be vastly different from clinical diagnostic imaging
1223 output. Clinical scanners can indeed be modified to be able to generate drug delivery protocols.

1224 In a similar way that elastography requires long ultrasound pulses to generate the push
1225 sequences (Deffieux, et al. 2009) , ultrasound scanners can be modified to be able to transmit
1226 drug delivery ultrasound sequences with tailored and optimized parameters (pulse duration,
1227 duty cycle, and center frequency).

1228 Ultimately, ultrasound image-guided drug delivery and the monitoring of treatment
1229 response could be feasible with the same equipment. Additionally, with recent developments
1230 in ultrasound imaging technology, ultrasound-mediated therapy could be planned, applied and
1231 monitored in a rapid sequence with high spatial and temporal resolution. The use of a single
1232 imaging and therapy device would alleviate the need for co-registration, because the imaging
1233 equipment would also be used to induce localized therapy ensuring a perfect co-location.
1234 Nonetheless, a compromise between efficacy and safety remains a major challenge for
1235 successful clinical applications of this dual methodology, which combines real-time image
1236 guidance of therapeutic delivery.

1237 In conclusion, ultrasound-responsive microbubbles which serve as cavitation nuclei are
1238 being used to treat a wide variety of diseases and show great potential preclinically and
1239 clinically. The elucidation of the cavitation nuclei – cell – interaction and the implementation
1240 of drug delivery ultrasound sequences on clinical ultrasound scanners are expected to
1241 invigorate clinical studies.

1242

1243 **ACKNOWLEDGEMENTS**

1244 Financial support from the European Research Council (ERC) under the European Union's
1245 Horizon 2020 research and innovation programme (grant agreement No 805308; PI: KK),
1246 Phospholipid Research Center in Heidelberg, Germany (PhD grant; PI: KK), FWO Vlaanderen
1247 (grant 12E3916N), U.S. Department of Health and Human Services, National Institutes of
1248 Health, National Institute of Neurological Disorders and Stroke grant R01 NS047603 (PI:

1249 CKH), Engineering and Physical Sciences Research Council (grants EP/I021795/1 and
1250 EP/L024012/1; PI: ES), the Canada Research Chair Program (PI: KH, PI: MAO) is gratefully
1251 acknowledged.

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2193

2194 **FIGURE CAPTIONS LIST**

2195 **Figure 1.** Combined effect of nonlinear propagation and focusing of the harmonics in a
2196 perfluoropentane micrometer-sized droplet. The emitted ultrasound wave has a frequency of
2197 3.5 MHz and a focus at 3.81 cm, and the radius of the droplet is 10 μm for ease of observation.
2198 The pressures are given on the axis of the droplet along the propagating direction of the
2199 ultrasound wave, and the shaded area indicates the location of the droplet (reprinted with
2200 permission from Sphak et al. (2014)).

2201

2202 **Figure 2.** Ultrasound-activated microbubbles can locally alter the tumor microenvironment
2203 through four mechanisms: enhanced permeability, improved contact, reduced hypoxia, and
2204 altered perfusion.

2205

2206 **Figure 3.** Schematic overview of how microbubbles and ultrasound have been shown to
2207 contribute to cancer immunotherapy. From left to right: microbubbles can be used as antigen
2208 carriers to stimulate antigen uptake by dendritic cells. Microbubbles and ultrasound can alter
2209 the permeability of tumors thereby increasing the intratumoral penetration of adoptively
2210 transferred immune cells or checkpoint inhibitors. Finally, exposing tissues to cavitating
2211 microbubbles can induce sterile inflammation by the local release of DAMPS.

2212

2213 **Figure 4.** 3D transcranial subharmonic microbubble imaging and treatment control *in vivo* in
2214 rabbit brain during BBB opening. Spectral information (top) shows the appearance of
2215 subharmonic activity at $t = 35$ s into the treatment. Passive mapping of the subharmonic band
2216 localizes this activity to the target region. Scale bar indicates 2.5 mm (reprinted (adapted) with
2217 permission from Jones et al. (2018)).

2218

2219 **Figure 5.** T₁ weighted sagittal MR images showing leptomeningeal tumors in rat spinal cord
2220 (grey arrowheads) before ultrasound and microbubble treatment (left column), and the
2221 enhancement of the cord indicating BSCB opening (white arrows) post-ultrasound and
2222 microbubble treatment (right column) (reprinted (adapted) with permission from O'Reilly et
2223 al. (2018)).

2224

2225 **Figure 6.** Simulated acoustic pressure and temperature in a representative subject exposed to
2226 pulsed 220 kHz ultrasound with a 33.3% duty cycle. The absolute peak-to-peak pressure
2227 maximum for the simulations is displayed in gray scale. Temperature is displayed using a heat
2228 map with a minimum color priority write threshold of 1 °C. Computed tomography features
2229 such as bone (cyan), skin and internal epithelium (beige), and clot (green), are plotted using
2230 contour lines. The transducer is outlined in magenta. Constructive interference is prominent in
2231 the soft tissue between the temporal bone and the transducer. Some constructive interference
2232 is also present in the brain tissue close to the contralateral temporal bone, however, the pressure
2233 in this region did not exceed the pressure in the M1 section of the middle cerebral artery.
2234 Temperature rise was prominent in the ipsilateral bone along the transducer axis.
2235 Computational model is described in Kleven et al. (2019).

2236

2237 **Figure 7.** Histological sections of a coronary artery of a pig 28 days after angioplasty. Pigs
2238 were treated with sirolimus-loaded microbubbles only (a) or sirolimus-loaded microbubbles
2239 and ultrasound (b) using a mechanically rotating intravascular ultrasound catheter (5 MHz, 500
2240 cycles, 50% duty cycle, 0.6 MPa peak negative pressure). Treatment with ultrasound and
2241 sirolimus-loaded microbubbles reduced neointimal formation by 50%. In both sections the
2242 intima (I) and media (M) are outlined; scale bar is 500 μm (Reprinted by permission from
2243 Springer Nature: Springer, *Annals of Biomedical Engineering, Reducing Neointima*
2244 *Formation in a Swine Model with IVUS and Sirolimus Microbubbles*, Kilroy JP, Dhanaliwala
2245 AH, Klibanov AL, Bowles DK, Wamhoff BR, Hossack JA, COPYRIGHT (2015)).

2246

2247 **Figure 8.** Different time scales of the therapeutic effects of ultrasound and cavitation nuclei
2248 treatment. $[\text{Ca}^{2+}]_i$ = intracellular calcium; ROS = reactive oxygen species; ATP = adenosine

2249 triphosphate; EV = extracellular vesicles (reprinted (adapted) with permission from Lattwein
2250 et al. (2019)).

1 **Ultrasound-responsive cavitation nuclei for therapy and drug delivery**

2

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36 **ABSTRACT**

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

51

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

54 INTRODUCTION

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

74

75 CAVITATION NUCLEI FOR THERAPY

76 The most widely used cavitation nuclei are phospholipid-coated microbubbles with a gas
77 core. For the 128 preclinical studies included in the treatment sections of this review, the
78 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.

104 (2015), Dong et al. (2017), Goyal et al (2017), Pandit et al. (2019). These phospholipids are
105 popular because they are also the main component in Definity[®] (Definity[®] 2011) and
106 SonoVue[®]/Lumason[®] (Lumason[®] 2016), respectively. Another key component of the
107 microbubble coating is a PEGylated emulsifier such as polyoxyethylene (40) stearate (PEG40-
108 stearate; for example Kilroy et al. (2014)) or the most often used 1,2-distearoyl-sn-glycero-3-
109 phosphoethanolamine-N-carboxy (poly-ethyleneglycol) (DSPE-PEG2000; for example Belcik
110 et al. (2017)), which is added to inhibit coalescence and to increase the *in vivo* half-life (Ferrara,
111 et al. 2009). In general two methods are used to produce custom-made microbubbles:
112 mechanical agitation (for example Ho et al. (2018)) or probe sonication (for example Belcik et
113 al. (2015)). Both these methods produce a population of microbubbles that is polydisperse in
114 size. Monodispersed microbubbles produced by microfluidics have recently been developed,
115 and are starting to gain attention for pre-clinical therapeutic studies. Dixon et al. (2019) used
116 monodisperse microbubbles to treat ischemic stroke.

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

129 with genetic material loaded on the microbubble surface by charge-coupling (for example Cao
130 et al. (2015)). Besides phospholipids and nanoparticles, microbubbles can also be coated with
131 denatured proteins such as albumin. Optison™ (Optison™ 2012) is a commercially available
132 and clinically approved ultrasound contrast agent that is coated with human albumin and used
133 in studies on treatment of non-brain tumors (Xiao, et al. 2019), BBB opening (Kovacs, et al.
134 2017b, Payne, et al. 2017), and immunotherapy (Maria, et al. 2015). Nano-sized particles cited
135 in this review have been used as cavitation nuclei for treatment of tumors, such as nanodroplets
136 (for example Cao et al. (2018)) and nanocups (Myers, et al. 2016), for BBB opening
137 (nanodroplets, Wu et al. (2018)), and for sonobactericide (nanodroplets, Guo et al. (2017a)).

138

139 **BUBBLE-CELL INTERACTION**

140 **Physics**

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

143 *Physics of Microbubble – Cell Interaction*

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

151 a. Mechanical effects

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

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

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

161

$$162 \quad \ddot{R} = -\left(\frac{3}{2}\frac{\dot{R}^2}{R}\right) + \left(\frac{p_G(R)+p_\infty(t)-\frac{2\sigma}{R}}{\rho_L R}\right) = IF + PF, \quad (\text{Eq. 1})$$

163

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

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

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

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

178 deforming fragile structures such as a biological cell membranes (van Wamel, et al. 2006, Kudo
179 2017) or blood vessel walls (Chen, et al. 2011).

180 (ii) Ballistic motion – in addition to oscillating, the bubble may undergo translation as a
181 result of the pressure gradient in the fluid generated by a propagating ultrasound wave (primary
182 radiation force). Due to their high compressibility, bubbles may travel at significant velocities,
183 sufficient to push them toward targets for improved local deposition of a drug (Dayton, et al.
184 1999) or penetrate biological tissue (Caskey, et al. 2009, Bader, et al. 2015, Acconcia, et al.
185 2016).

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

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

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

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

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

217 b. Thermal effects

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

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

231 c. Chemical effects

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

240

241 *Physics of Droplets – Cell Interaction*

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

278 larger than the applied wavelength. In the current case, the frequency of a typical ultrasound
279 wave used for insonification is in the order of 1-5 MHz, yielding wavelengths in the order of
280 1500 – 300 μm , while a droplet will be smaller by 2-4 orders of magnitude. Beside this, using
281 the ray approach, the lower speed of sound in perfluorocarbon would yield a focal spot near
282 the backside of the droplet, which is in contradiction to observations. The correct way to treat
283 the focusing effect is to solve the full diffraction problem by decomposing the incident wave,
284 the wave reflected by the droplet, and the wave transmitted into the droplet into a series of
285 spherical waves. For each spherical wave, the spherical reflection and transmission coefficients
286 can be derived. Superposition of all the spherical waves yields the pressure inside the droplet.
287 Nevertheless, when this approach is only applied to an incident wave with the frequency that
288 is emitted by the transducer, this will lead neither to the right nucleation spot nor to sufficient
289 negative pressure for vaporization. Nanoscale droplets may be too small to make effective use
290 of the focusing mechanism and ADV is therefore less dependent on the frequency.

291

292 b. Nonlinear ultrasound propagation

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

$$299 \quad p(t) = \sum_{n=1}^{\infty} a_n \cos(n\omega t + \phi_n), \quad (\text{Eq. 2})$$

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

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

312 After vaporization has started, the growth of the emerging bubble is limited by inertia and
313 heat transfer. In the absence of the heat transfer limitation, the inertia of the fluid that surrounds
314 the bubble limits the rate of bubble growth, which is linearly proportional to time and inversely
315 proportional to the square root of the density of the surrounding fluid. When inertia is
316 neglected, thermal diffusion is the limiting factor in the transport of heat to drive the
317 endothermic vaporization process of perfluorocarbon, causing the radius of the bubble to
318 increase with the square root of time. In reality, both processes occur simultaneously, where
319 the inertia effect is dominant at the early stage and the diffusion effect is dominant at the later
320 stage of bubble growth. The final size that is reached by a bubble depends on the time that a
321 bubble can expand, i.e. on the duration of the negative cycle of the insonifying pressure wave.
322 It is therefore expected that lower insonification frequencies give rise to larger maximum
323 bubble size. Thus, irrespective of their influence on triggering ADV, lower frequencies would
324 lead to more violent inertial cavitation effects and cause more biological damage, as
325 experimentally observed for droplets with a radius in the order of 100 nm (Burgess and Porter
326 2019).

327

328 **Biological mechanisms and bioeffects of ultrasound-activated cavitation nuclei**

329 The biological phenomena of sonoporation (*i.e.* membrane pore formation), stimulated
330 endocytosis, and opening of cell-cell contacts and the bioeffects of intracellular calcium
331 transients, reactive oxygen species generation, cell membrane potential change, and
332 cytoskeleton changes have been **observed** for several years (Sutton, et al. 2013, Kooiman, et
333 al. 2014, Lentacker, et al. 2014, Qin, et al. 2018b). However, other bioeffects induced by
334 ultrasound-activated cavitation nuclei have recently been discovered. These include membrane
335 blebbing as a recovery mechanism for reversible sonoporation (both for ultrasound-activated
336 microbubbles (Leow, et al. 2015) and upon ADV (Qin, et al. 2018a)), extracellular vesicle
337 formation (Yuana, et al. 2017), suppression of efflux transporters P-glycoprotein (Cho, et al.
338 2016, Aryal, et al. 2017) and BBB (Blood Brain Barrier) transporter genes (McMahon, et al.
339 2018). At the same time, more insight has been gained in the **origin of the bioeffects**, largely
340 through the use of live cell microscopy. For sonoporation, real time membrane pore opening
341 and closure dynamics were revealed with pores $<30 \mu\text{m}^2$ closing within 1 min, while pores
342 $>100 \mu\text{m}^2$ did not reseal (Hu, et al. 2013) as well as immediate rupture of filamentary actin at
343 the pore location (Chen, et al. 2014) and correlation of intracellular reactive oxygen species
344 levels with the degree of sonoporation (Jia, et al. 2018). Real-time sonoporation and opening
345 of cell-cell contacts in the same endothelial cells has been demonstrated as well for a single
346 example (Helfield, et al. 2016). The applied acoustic pressure was shown to determine uptake
347 of model drugs via sonoporation or endocytosis in another study (De Cock, et al. 2015).
348 Electron microscopy revealed formation of transient membrane disruptions and permanent
349 membrane structures, *i.e.* caveolar endocytic vesicles, upon ultrasound and microbubble-
350 treatment (Zeghimi, et al. 2015). A study by Fekri et al. (2016) revealed that enhanced clathrin-
351 mediated endocytosis and fluid-phase endocytosis occur through distinct signaling
352 mechanisms upon ultrasound and microbubble treatment. The majority of these **bioeffects** have

353 been observed in *in vitro* models using largely non-endothelial cells and may therefore not be
354 directly relevant to *in vivo* tissue, where intravascular micron-sized cavitation nuclei will only
355 have contact with endothelial cells and circulating blood cells. On the other hand, the
356 mechanistic studies by Belcik et al. (2015, 2017) and Yu et al. (2017) do show translation from
357 *in vitro* to *in vivo*. In these studies, ultrasound-activated microbubbles were shown to induce a
358 shear-dependent increase in intravascular adenosine triphosphate (ATP) from both endothelial
359 cells and erythrocytes, an increase in intramuscular nitric oxide, and downstream signaling
360 through both nitric oxide and prostaglandins which resulted in augmentation of muscle blood
361 flow. Ultrasound settings were similar, namely 1.3 MHz, MI 1.3 for Belcik et al. (2015, 2017)
362 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
363 negative pressure of the ultrasound wave (in MPa) and f the center frequency of the ultrasound
364 wave (in MHz).

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

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

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

383

384 **Modelling Microbubble – cell – drug interaction**

385 Whilst there have been significant efforts to model the dynamics of ultrasound driven
386 microbubbles (Faez, et al. 2013, Dollet, et al. 2019), less attention has been paid to the
387 interactions between microbubbles and cells or their impact upon drug transport. Currently
388 there are no models that describe the interactions between microbubbles, cells, and drug
389 molecules. Several models have been proposed for the microbubble – cell interaction in
390 sonoporation focusing on different aspects: the cell expansion and microbubble jet velocity
391 (Guo, et al. 2017b), the shear stress exerted on the cell membrane (Wu 2002, Doinikov and
392 Bouakaz 2010, Forbes and O'Brien 2012, Yu and Chen 2014, Cowley and McGinty 2019),
393 microstreaming (Yu and Chen 2014), shear stress exerted on the cell membrane **in combination**
394 **with** microstreaming (Li, et al. 2014), or other flow phenomena (Yu, et al. 2015, Rowlatt and
395 Lind 2017) generated by an oscillating microbubble. In contrast to the other models, Man et al.
396 (2019) propose that the microbubble-generated shear stress does not induce pore formation,
397 but that this is instead due to microbubble fusion with the membrane and subsequent “pull out”
398 of cell membrane lipid molecules by the oscillating microbubble. Models for pore formation
399 (for example Koshiyama and Wada (2011)) and resealing (Zhang, et al. 2019) in cell
400 membranes have also been developed, but these models neglect the mechanism by which the
401 pore is created. There is just one sonoporation dynamics model, developed by Fan *et al.* (2012),
402 that relates the uptake of the model drug propidium iodide (PI) to the size of the created

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

$$405 \quad F(t) = \alpha \cdot \pi D C_0 \cdot r_0 \cdot \frac{1}{\beta} (1 - e^{-\beta t}), \quad (\text{Eq. 3})$$

406 where α is the coefficient that relates the amount of PI molecules to the fluorescence intensity
407 of PI-DNA and PI-RNA, D is the diffusion coefficient of PI, C_0 is the extracellular PI
408 concentration, r_0 is the initial radius of the pore, β is the pore resealing coefficient, and t is
409 time. The coefficient α is determined by the sensitivity of the fluorescence imaging system,
410 and if unknown the equation can still be used because it is the pore size coefficient, $\alpha \cdot \pi D C_0 \cdot r_0$,
411 that determines the initial slope of the PI uptake pattern and is the scaling factor for the
412 exponential increase. A cell with a large pore will have a steep initial slope of PI uptake and
413 the maximum PI intensity quickly reaches the plateau value. A limitation of this model is that
414 equation 3 is based on two-dimensional free diffusion models, which holds for PI-RNA but not
415 for PI-DNA because this is confined to the nucleus. The model is independent of cell type, as
416 Fan et al. have demonstrated agreement with experimental results in both kidney (Fan, et al.
417 2012) and endothelial cells (Fan, et al. 2013). Other researchers have also used this model for
418 endothelial cell studies and also classified the distribution of both the pore size and pore
419 resealing coefficients using Principal Component Analysis to determine whether cells were
420 reversibly or irreversibly sonoporated. In the context of blood brain barrier (BBB) opening,
421 Hosseinkhah et al. (2015) have modeled the microbubble-generated shear and circumferential
422 wall stress for 5 μm microvessels upon microbubble oscillation at a fixed mechanical index
423 (MI) of 0.134 for a range of frequencies (0.5, 1, and 1.5 MHz). The wall stresses were
424 dependent upon microbubble size (range investigated 2 – 18 μm in diameter) and ultrasound
425 frequency. Wiedemair et al. (2017) have also modelled the wall shear stress generated by
426 microbubble (2 μm diameter) destruction at 3 MHz for larger microvessels (200 μm diameter).
427 The presence of red blood cells was included in the model and was found to cause confinement

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

431 .

432

433 TREATMENT OF TUMORS (NON-BRAIN)

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

442 Notwithstanding the growing knowledge of the pathophysiology of tumors, treatment
443 remains challenging. Chemotherapeutic drugs are typically administered to abolish the rapidly-
444 dividing cancer cells. Yet, their cytotoxic effects are not limited to cancer cells, causing dose-
445 limiting off-target effects. To overcome this hurdle, chemotherapeutics are often encapsulated
446 in nano-sized carriers, i.e. nanoparticles, that are designed to specifically diffuse through the
447 large openings of tumor vasculature, while being excluded from healthy tissue by normal blood
448 vessels (Lammers, et al. 2012, Maeda 2012). Despite being highly promising in pre-clinical
449 studies, drug-containing nanoparticles have shown limited clinical success due to the vast
450 heterogeneity in tumor vasculature (Barenholz 2012, Lammers, et al. 2012, Wang, et al.
451 2015d). In addition, drug penetration into the deeper layers of the tumor can be constrained
452 due to high interstitial pressure and a dense extracellular matrix in the tumor. Furthermore,

453 acidic and hypoxic regions limit the efficacy of radiation- and chemotherapy-based treatments
454 due to biochemical effects (Mehta, et al. 2012, McEwan, et al. 2015, Fix, et al. 2018).
455 Ultrasound-triggered microbubbles are able to alter the tumor environment locally, thereby
456 improving drug delivery to tumors. These alterations are schematically represented in Figure
457 2 and include: improving vascular permeability, modifying the tumor perfusion, reducing local
458 hypoxia, and overcoming the high interstitial pressure.

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

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

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

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

500 Apart from their ability to improve the tumor uptake, microbubbles can be used as
501 ultrasound-responsive drug carriers to reduce the off-target effects of chemotherapeutics. By
502 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, Kotopoulos, 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

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

550

551 **Clinical studies**

552 *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 (Kotopoulos, et al. 2013,
556 Dimceovski, et al. 2016). In this clinical trial, gemcitabine was administered 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,

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

585

586 *Hepatic metastases from digestive system*

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

603 In addition, the severity of side effects did not worsen with increases in MI, suggesting that
604 microbubble-mediated chemotherapy is a safe procedure. The secondary aims were to assess
605 the efficacy of this therapeutic protocol using contrast-enhanced CT and MRI. Thus, tumor
606 size and development were characterized according to the RECIST criteria. Half of the patients
607 had stable disease and one patient obtained a partial response after the first treatment cycle.
608 The median progression-free survival was 91 days. However, making any comparison and
609 interpretation of results is very difficult because none of the patients were treated with the same
610 chemotherapeutics, MI, and/or number of treatment cycles. The results of safety and efficacy
611 evaluations should be compared to patients receiving the chemotherapy on its own in order to
612 clearly identify the therapeutic benefit of combining with ultrasound-driven microbubbles.
613 Similar to the pancreatic clinical study, no direct evidence of enhanced therapeutic
614 bioavailability of the chemotherapeutic drug after the treatment was provided. This
615 investigation is all the more important as the ultrasound and microbubble treatment was applied
616 30 min after intravenous chemotherapy (for both monotherapy and combination therapy)
617 independently of drug pharmacokinetics and metabolism.

618

619 *Ongoing and upcoming clinical trials*

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

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

641

642 IMMUNOTHERAPY

643 Cancer immunotherapy is considered to be one of the most promising strategies to eradicate
644 cancer as it makes use of the patient's own immune system to selectively attack and destroy
645 tumor cells. It is a common name that refers to a variety of strategies that aim to unleash the
646 power of the immune system by either boosting antitumoral immune responses or flagging
647 tumor cells to make them more visible to the immune system. The principle is based on the
648 fact that tumors express specific tumor antigens which are not, or to a much lesser extent,
649 expressed by normal somatic cells and hence can be used to initiate a cancer-specific immune
650 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

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

691 Another strategy in cancer immunotherapy is adoptive cell therapy, where *ex vivo*
692 manipulated immune effector cells, mainly T cells and NK (natural killer) cells, are employed
693 to generate a robust and selective anticancer immune response (Yee 2018, Hu, et al. 2019).
694 These strategies have mainly led to successes in hematological malignancies, not only because
695 of the availability of selective target antigens, but also because of the accessibility of the
696 malignant cells (Khalil, et al. 2016, Yee 2018). By contrast, in solid tumors, and especially in
697 brain cancers, inadequate homing of cytotoxic T cells or NK cells to the tumor proved to be
698 one of the main reasons for the low success rates, making the degree of tumor infiltration an
699 important factor in disease prognosis (Childs and Carlsten 2015, Gras Navarro, et al. 2015, Yee
700 2018). To address this, focused ultrasound and microbubbles have been used to make tumors

701 more accessible to cellular therapies. The first demonstration of this concept was provided by
702 Alkins et al. (2013) who used a xenograft HER-2-expressing breast cancer brain metastasis
703 model to determine whether ultrasound and microbubbles could allow intravenously infused
704 NK cells to cross the blood-brain barrier (BBB). By loading the NK cells with
705 superparamagnetic iron oxide (SPIO) nanoparticles, the accumulation of NK cells in the brain
706 could be tracked and quantified via MRI. An enhanced accumulation of NK cells was found
707 when the cells were injected immediately prior to BBB disruption. Importantly NK cells
708 retained their activity and ultrasound treatment resulted in a sufficient NK to tumor cell ratio
709 to allow effective tumor cell killing (Alkins, et al. 2016). In contrast, very few NK cells reached
710 the tumor site when BBB disruption was absent or performed before NK cell infusion.
711 Although it is not known for certain why timing had such a significant impact on NK
712 extravasation, it is likely that the most effective transfer to the tissue occurs at the time of
713 insonification, and that the barrier is most open during this time (Marty, et al. 2012). Possible
714 other explanations include the difference in size of the temporal BBB openings or a possible
715 alternation in the expression of specific leukocyte adhesion molecules by the BBB disruption,
716 thus facilitating the translocation of NK cells. Also for tumors where BBB crossing is not an
717 issue, ultrasound has been used to improve delivery of cellular therapeutics. Sta Maria et al.
718 (2015) demonstrated enhanced tumor infiltration of adoptively transferred NK cells after
719 treatment with microbubbles and low dose focused ultrasound. This result was confirmed by
720 Yang et al. (2019a) in a more recent publication where the homing of NK cells was more than
721 doubled after microbubble injection and ultrasound treatment of an ovarian tumor. Despite the
722 enhanced accumulation, however, the authors did not observe an improved therapeutic effect,
723 which might be due to the limited number of treatments that were applied, or the
724 immunosuppressive tumor microenvironment that counteracts the cytotoxic action of the NK
725 cells.

726 There is growing interest in exploring the effect of microbubbles and ultrasound on the
727 tumor microenvironment, as recent work has shown that BBB disruption with microbubbles
728 and ultrasound may induce sterile inflammation. Although a strong inflammatory response may
729 be detrimental in the case of drug delivery across the BBB, it might be interesting to further
730 study this inflammatory response in solid tumors as it might induce the release of damage-
731 associated molecular patterns (DAMPS) such as heat-shock proteins and inflammatory
732 cytokines. This could shift the balance towards a more inflammatory microenvironment that
733 could promote immunotherapeutic approaches. As reported by Liu *et al.* (2012) exposure of a
734 CT26 colon carcinoma xenograft to microbubbles and low pressure pulsed ultrasound
735 increased cytokine release and triggered lymphocyte infiltration. Similar data have been
736 reported by Hunt *et al.* (2015). In their study, ultrasound treatment caused a complete shut-
737 down of tumor vasculature followed by the expression of HIF-1 α (hypoxia-inducible factor
738 1 α), a marker of tumor ischemia and tumor necrosis, as well as increased infiltration of T cells.
739 Similar responses have been reported following thermal and mechanical HIFU treatments of
740 solid tumors (Unga and Hashida 2014, Silvestrini, et al. 2017). A detailed review of ablative
741 ultrasound therapies is however out of the scope of this review.

742 At present, the most successful form of immunotherapy is the administration of monoclonal
743 antibodies to inhibit regulatory immune checkpoints that block T cell action. Examples are
744 CTLA-4 (cytotoxic T lymphocyte-associated protein-4) and PD-1 (programmed cell death-1),
745 which act as brakes on the immune system. Blocking the effect of these brakes can revive and
746 support the function of immune effector cells. Despite the numerous successes achieved with
747 checkpoint inhibitors, responses have been quite heterogeneous as the success of checkpoint
748 inhibition therapy largely depends on the presence of intratumoral effector T cells (Weber
749 2017). This motivated Bulner et al. (2019) to explore the synergy of microbubble and
750 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.

776

777 **Mechanisms, Bioeffects, and Safety**

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

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

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

801 exogenous therapeutics. These effects include a reduction in amyloid- β plaque load (Jordão, et
802 al. 2013, Burgess, et al. 2014, Leinenga and Götz 2015, Poon, et al. 2018), reduction in tau
803 pathology (Pandit, et al. 2019), and improvements in spatial memory (Burgess, et al. 2014,
804 Leinenga and Götz 2015). Two-photon microscopy has shown that amyloid- β plaque size is
805 reduced in transgenic mice for up to two weeks post ultrasound and microbubble treatment
806 (Poon, et al. 2018). Opening of the BBB in both transgenic and wild-type mice has also
807 revealed enhanced neurogenesis (Burgess, et al. 2014, Scarcelli, et al. 2014, Mooney, et al.
808 2016) in the treated tissue.

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

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

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

831

832 **Cavitation Monitoring and Control**

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

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

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

863

864 **BSCB opening**

865 Despite the similarities between the BBB and BSCB, and the great potential benefit for
866 patients, there has been limited work investigating translation of this technology to the spinal
867 cord. Opening of the BSCB in rats was first reported by Wachsmuth et al. (2009), and was
868 followed by studies from Weber-Adrien et al. (2015), Payne et al. (2017), and O'Reilly et al.
869 (2018) in rats (Figure 5) and from Montero et al. (2019) in rabbits, the latter performed through
870 a laminectomy window. In 2018, O'Reilly et al. (2018) presented the first evidence of a
871 therapeutic benefit in a disease model, showing improved tumor control in a rat model of
872 leptomeningeal metastases.

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

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

883

884 **Clinical studies**

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

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

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

904 Another study conducted in Alzheimer's disease patients with the Exablate device
905 demonstrated safe BBB permeability enhancement and that the treatment could be repeated
906 one month later without any imaging or behavior indications of adverse events (Lipsman, et al.
907 2018). A third study with the same device investigated the feasibility of using functional MRI
908 to target motor cortex in Amyotrophic Lateral Sclerosis (ALS) patients again showing precisely
909 targeted BBB permeability enhancement without adverse effects in this delicate structure
910 (Abraham, et al. 2019). All of these studies were conducted using Definity[®] microbubbles.
911 These studies have led to the current ongoing brain tumor trial with six monthly treatments of
912 the brain tissue surrounding the resection cavity during the maintenance phase of the treatment
913 with temozolomide. This study sponsored by InSightec is being conducted in multiple
914 institutions. Similarly, a phase II trial in Alzheimer's disease sonicating the hippocampus with
915 the goal of investigating the safety and potential benefits from repeated (three treatments with
916 two-week interval) BBB permeability enhancement alone is ongoing. This study is also being
917 conducted in several institutions that have the device.

918

919 **SONOTROMBOLYSIS**

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

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

930

931 **Mechanisms, Agents, and Approaches**

932 Ultrasound improves recombinant tissue plasminogen activator (rt-PA) diffusion into
933 thrombi and augments lysis primarily via acoustic radiation force and streaming (Datta, et al.
934 2006, Prokop, et al. 2007, Petit, et al. 2015). Additionally, ultrasound increases rt-PA and
935 plasminogen penetration into the thrombus surface and enhances removal of fibrin degradation
936 products via ultrasonic bubble activity, or acoustic cavitation, that induces microstreaming
937 (Elder 1958, Datta, et al. 2006, Sutton, et al. 2013). Two types of cavitation are correlated with
938 enhanced thrombolysis: stable cavitation, with highly nonlinear bubble motion resulting in
939 acoustic emissions at the subharmonic and ultraharmonics of the fundamental frequency (Flynn
940 1964, Phelps and Leighton 1997, Bader and Holland 2013), and inertial cavitation, with
941 substantial radial bubble growth and rapid collapse generating broadband acoustic emissions
942 (Carstensen and Flynn 1982, Flynn 1982).

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

951 shelled microbubbles (Weller, et al. 2002) as a nucleation agent. However, Kleven et al. (2019)
952 observed more than 60% fractional clot width loss for highly retracted human whole blood
953 clots exposed to rt-PA, Definity[®] and 220 kHz pulsed or continuous wave (CW) ultrasound at
954 an acoustic output with sustained stable cavitation throughout the insonification periods
955 (0.22 MPa peak rarefactional pressure) (Figure 6).

956 Echogenic liposomes loaded with rt-PA enhanced lysis compared to rt-PA alone at
957 concentrations of 1.58 and 3.15 mg/mL (Shekhar, et al. 2017), suggesting that encapsulation
958 of rt-PA could reduce the rt-PA dose by a factor of two with equivalent lytic activity.
959 Subsequently it has been demonstrated that these liposomes protect rt-PA against degradation
960 by plasminogen activator inhibitor-1 (PAI-1), while achieving equivalent thrombolytic
961 efficacy relative to rt-PA, Definity[®], and intermittent 220 kHz CW ultrasound (Shekhar, et al.
962 2019). Promising agents, including a nanoscale (< 100 nm) contrast agent (Brüssler, et al.
963 2018) and magnetically targeted microbubbles (De Saint Victor, et al. 2019), have also
964 demonstrated enhanced rt-PA thrombolysis *in vitro*. All of these investigators noted that in the
965 absence of rt-PA, the combination of ultrasound and microbubbles did not degrade the fibrin
966 network.

967 Several minimally invasive techniques have also been explored, with or without the
968 inclusion of rt-PA or exogenous cavitation nuclei. In the clinical management of stroke, rapid
969 treatments are needed because of the neurologist's adage "time is brain". Thus, treatment
970 options that promote fast clot removal, reduce edema and intracerebral bleeding, and improve
971 patient outcomes are of immense value. Magnetic resonance image-guided high intensity
972 focused ultrasound has been investigated for the treatment of both ischemic (Burgess, et al.
973 2012) and hemorrhagic (Monteith, et al. 2013) stroke, and Zafar et al. (2019) have provided an
974 excellent review of the literature for this approach. Histotripsy, a form of high intensity focused
975 ultrasound that relies on the mechanical action of microbubble clouds to ablate thrombi with

976 and without rt-PA (Maxwell, et al. 2009, Bader, et al. 2015, Zhang, et al. 2016b, Bader, et al.
977 2019) is under development to treat deep vein thrombosis. Additionally, ultrasound-accelerated
978 catheter-directed thrombolysis using the EKOS system (EKOS/BTG, Bothell, WA, USA)
979 combines 2 MHz low-intensity pulsed ultrasound and rt-PA without cavitation nuclei to
980 improve lytic efficiency to treat deep vein thrombosis (Shi, et al. 2018) and pulmonary
981 embolism (Garcia 2015).

982

983 **Cavitation monitoring**

984 Acoustic cavitation has been shown to mediate direct fibrinolysis (Weiss, et al. 2013) and
985 accelerated rt-PA lysis (Everbach and Francis 2000, Datta, et al. 2006, Prokop, et al. 2007,
986 Hitchcock, et al. 2011). Passive and active cavitation detection techniques have been developed
987 to monitor acoustic cavitation (Roy, et al. 1990, Madanshetty, et al. 1991, Bader, et al. 2015).
988 Passive cavitation imaging, or passive acoustic mapping, employs a transducer array that
989 listens passively (i.e., no transmit) to emissions from acoustically activated microbubbles
990 (Salgaonkar, et al. 2009, Gyöngy and Coussios 2010, Haworth, et al. 2017). Vignon et al.
991 (2013) developed a prototype array enabling spectral analysis of bubble activity for
992 sonothrombolysis applications. Superharmonic Doppler effects have also been utilized to
993 monitor bubble activity from 500 kHz pulsed therapeutic ultrasound (Pouliopoulos and Choi
994 2016). Both a linear array (Arvanitis and McDannold 2013a, Arvanitis, et al. 2013, Arvanitis
995 and McDannold 2013b) and a sparse hemispherical array (Acconcia, et al. 2017) have been
996 integrated into a clinical magnetic resonance image-guided high intensity focused ultrasound
997 system to assess microbubble dynamics during sonothrombolysis in the brain.

998

999 **Preclinical studies**

1000 Information gathered from animal studies can help inform human clinical trials, despite a
1001 strong species dependence of clot rt-PA lytic susceptibility (Gabriel, et al. 1992, Flight, et al.
1002 2006, Huang, et al. 2017). A comprehensive systematic evaluation of 16 *in vivo* preclinical
1003 sonothrombolysis studies was carried out by Auboire et al. (2018) summarizing treatment
1004 efficacy and safety outcomes in models of ischemic stroke. Since that review was published,
1005 the efficacy of sonothrombolysis using nitrogen microbubbles stabilized with a non-
1006 crosslinked shell delivered intra-arterially through a catheter and rt-PA delivered intravenously
1007 has been demonstrated in a rat model of ischemic stroke (Dixon, et al. 2019).

1008

1009 **Clinical studies**

1010 A rich literature exists of clinical trials exploring the safety and efficacy of
1011 sonothrombolysis. Two recent meta-analyses of seven randomized controlled trials (Chen, et
1012 al. 2019, Zafar, et al. 2019) attempt to determine whether the administration of rt-PA and
1013 ultrasound improve outcomes in acute ischemic stroke. Both analyses conclude that
1014 sonothrombolysis significantly enhances complete or partial recanalization, with improved
1015 neurologic function (assessed via the National Institutes of Health Stroke Scale, NIHSS). An
1016 ongoing clinical trial (TRUST; NCT03519737) will determine whether large vessel occlusions
1017 can be recanalized with sonothrombolysis (Cerevast Medical, Inc., Bothell, WA, USA) and rt-
1018 PA, tenecteplase or alteplase, (Campbell, et al. 2018) while patients are transferred to a stroke
1019 center for mechanical thrombectomy (Gauberti 2019).

1020 Several clinical trials have shown that high MI pulsed diagnostic ultrasound exposure of
1021 Definity® before and after percutaneous coronary intervention for ST elevation myocardial
1022 infarction can prevent microvascular obstruction and improve functional outcomes (Mathias,
1023 et al. 2016, Mathias, et al. 2019, Slikkerveer, et al. 2019). A systematic review of 16 catheter-
1024 directed sonothrombolysis clinical trials comprised mostly of retrospective case series using

1025 the EKOS system without microbubble infusions determined that this treatment modality is
1026 safe and promising for the treatment of deep vein thrombosis, DVT (Shi, et al. 2018). However,
1027 a large-sample randomized prospective clinical trial is needed to improve the clinical evidence
1028 for use as a front-line therapy for DVT. In retrospective studies in patients with pulmonary
1029 embolism Lee et al. (2017) conclude that catheter directed sonothrombolysis is safe and
1030 decreases right-sided heart strain, but Schissler et al. (2018) conclude that this therapy is not
1031 associated with a reduction in mortality nor increased resolution of right ventricular
1032 dysfunction. And finally, an ongoing trial in a small cohort of 20 patients with acute peripheral
1033 arterial occlusions (Ebben, et al. 2017) will determine whether Luminity[®] (marketed in the US
1034 as Definity[®]) and 1.8 MHz transdermal diagnostic ultrasound with intermittent high MI (1.08)
1035 and low MI (0.11) for visualization of the microbubbles and flow will improve recanalization.
1036 In summary, sonothrombolysis has demonstrated clinical benefit in the treatment of acute and
1037 chronic thrombotic disease. Ultrasound-assisted thrombolysis has a potential role as an
1038 emerging viable and therapeutic option for future management of stroke and cardiovascular
1039 disease.

1040

1041 **CARDIOVASCULAR DRUG DELIVERY AND THERAPY**

1042 In cardiovascular drug delivery, cavitation nuclei are co-administered or loaded with
1043 different therapeutics for the treatment of various diseases. For atherosclerosis treatment in an
1044 ApoE-deficient mouse model, intercellular adhesion molecule-1 targeted microbubbles
1045 carrying angiogenesis inhibitor Endostar were used (Yuan, et al. 2018). Upon intermittent
1046 insonification over the abdominal and thoracic cavity with 1 MHz ultrasound (2 W/cm²
1047 intensity, 50% duty cycle) for 30 s with two repeats and another treatment 48 h later, plaque
1048 area and intraplaque neovascularization were significantly reduced two weeks after treatment.
1049 Percutaneous coronary intervention is often used to restore blood flow in atherosclerotic

1050 arteries. The treatment of coronary microembolization, a complication of percutaneous
1051 coronary intervention, was demonstrated in pigs **treated with** ultrasound (1 MHz, 2.0 W/cm²
1052 intensity, 10 s on and 10 s off, 20 min duration) and microRNA-21-loaded microbubbles four
1053 days before coronary microembolization (Su, et al. 2015). This resulted in an improved cardiac
1054 dysfunction. Although not a therapeutic study, Liu et al. (2015) did show that plasmid
1055 transfection to the myocardium was significantly larger when the microbubbles were
1056 administered into the coronary artery compared to intravenously via the ear vein in pigs even
1057 though the intracoronary microbubble dose was half of the intravenous dose (1 MHz
1058 ultrasound, 2 W/cm², 50% duty cycle, 20 min duration). Percutaneous coronary intervention
1059 can also result in neointimal formation which induces restenosis. Sirolimus-loaded
1060 microbubbles were shown to reduce neointimal formation in coronary arteries by 50% in pigs,
1061 see Figure 7, 28 days after angioplasty in combination with a mechanically rotating
1062 intravascular ultrasound catheter (5 MHz, 500 cycles, 50% duty cycle, 0.6 MPa peak negative
1063 pressure) (Kilroy, et al. 2015). Another research group showed that paclitaxel-loaded
1064 microbubbles and ultrasound (1 MHz, 1.5 MPa for 10 s) can also significantly inhibit
1065 neointimal formation in the iliac artery in rabbits one week after percutaneous coronary
1066 intervention (Zhu, et al. 2016).

1067 In diabetic cardiomyopathy, microbubble-mediated delivery of fibroblast growth factor has
1068 shown therapeutic effects. Zhao et al (2016) could prevent diabetic cardiomyopathy in rats by
1069 treating the heart with ultrasound (14 MHz, 7.1 MPa for 10 s, three repeats with off interval of
1070 1 s) and microbubbles co-administered with acidic fibroblast growth factor nanoparticles twice
1071 weekly for 12 consecutive weeks. In already established diabetic cardiomyopathy in rats, the
1072 same investigators co-administered basic fibroblast growth factor-containing nanoparticles
1073 with microbubbles with the same ultrasound treatment, albeit that it was given three times with
1074 one day in between treatments. At four weeks after treatment, this resulted in restored cardiac

1075 functions as a result of structural remodeling of the cardiac tissue (Zhao, et al. 2014).
1076 Microbubbles loaded with acidic fibroblast growth factor in combination with ultrasound (14
1077 MHz, 7.1 MPa for 10 s, three repeats with off interval of 1 s) also showed significantly
1078 improved cardiac function in a rat model of diabetic cardiomyopathy. Treatment was
1079 performed twice weekly for 12 consecutive weeks (Zhang, et al. 2016a). For doxorubicin
1080 induced cardiomyopathy, repeated co-administration of microbubbles and nanoparticles
1081 containing acidic fibroblast growth factor in combination with ultrasound (14 MHz, 7.1 MPa
1082 for 10 s, three repeats with off interval of 1 s) applied at the heart successfully prevented
1083 doxorubicin induced cardiomyopathy in rats (Tian, et al. 2017). Once doxorubicin induced
1084 cardiomyopathy had occurred, microbubble-mediated reversal of cardiomyopathy was shown
1085 by the delivery of survivin plasmid to cardiomyocytes and endothelial cells (Lee, et al. 2014)
1086 or glucagon-like peptide-1 (GLP-1) to cardiomyocytes, endothelial cells, vascular muscle cells,
1087 and mesenchymal cells (Chen, et al. 2015) in rats. The ultrasound settings were 5 MHz (120 V
1088 power, pulsing interval of 10 cardiac cycles at end-systole) for a 5 min treatment (Lee, et al.
1089 2014) or not specified (Chen, et al. 2015). The microbubble-mediated gene therapy study by
1090 Chen et al. (2016) showed that ANGPTL8 gene therapy does not need to be done in the heart
1091 to reverse doxorubicin induced cardiomyopathy in rats as their microbubble and ultrasound
1092 (1.3 MHz, 1.4 MPa peak negative pressure, four bursts triggered to every fourth end-systole
1093 using a delay of 45-70 ms of the peak of the R wave) therapy was done in the liver (90 s
1094 treatment). This resulted in overexpression of ANGPTL8 in liver cells and blood which
1095 stimulated cardiac progenitor cells in the epicardium.

1096 A few dozen articles have been published on treating myocardial infarction with
1097 microbubble and ultrasound-mediated gene delivery *in vivo*, in mouse, rat, rabbit, and dog
1098 models. These are reviewed by Qian et al. (2018). Amongst these are a few targeted
1099 microbubble studies which all show that the targeted microbubbles induced higher degrees of

1100 gene transfection, increased myocardial vascular density, and improved cardiac function in
1101 comparison to non-targeted microbubbles. This improvement occurred independent of the type
1102 of ligand on the microbubble, the gene that was transfected, or the animal model: matrix
1103 metalloproteinase 2 target with Timp3 gene in rats (Yan, et al. 2014), intracellular adhesion
1104 molecule-1 target with Ang-1 gene in rabbits (Deng, et al. 2015), P-selectin target with
1105 hVEGF165 gene in rats (Shentu, et al. 2018). Ultrasound settings for these studies were similar
1106 at 1.6 MHz (1.6 MPa peak negative pressure, pulsing interval of four cardiac cycles) for 20
1107 min during infusion of the plasmid-loaded microbubbles (both Yan et al. (2014) and Shentu et
1108 al. (2018)), or 1.7 MHz (1.7 MPa peak negative pressure, pulsing interval every four to eight
1109 cardiac cycles) for 5 min after bolus injection of the plasmid-loaded microbubbles (Deng, et
1110 al. 2015).

1111 Other gene therapy studies for vascular disease include stimulating angiogenesis for the
1112 treatment of chronic hindlimb ischemia in rats using miR-126-3p-loaded microbubbles and
1113 ultrasound (1.3 MHz, 2.1 MPa peak negative acoustic pressure, pulsing interval 5 s). The
1114 treatment lasted for 20 min of which microbubbles were infused for 10 min and resulted in
1115 improved perfusion, vessel density, arteriolar formation, and neovessel maturation (Cao, et al.
1116 2015). Recently, successful gene therapy was demonstrated in baboons where Vascular
1117 Endothelial Growth Factor (VEGF)-plasmid loaded microbubbles were infused and ultrasound
1118 (2-6 MHz, MI 1.9, repeated 5 s burst pulses with three bursts per minute) was applied for 10
1119 min on days 25, 35, 45, and 55 of gestation with the transducer placed over the placental basal
1120 plate (Babischkin, et al. 2019). This was a mechanistic study elucidating the role of VEGF in
1121 uterine artery remodeling.

1122 The gas core of the cavitation nuclei can also be the therapeutic. Sutton et al. (2014) have
1123 shown that ultrasound-mediated (1 MHz, 0.34 MPa acoustic pressure, 30 cycle pulse, 50 s
1124 treatment) nitric oxide gas delivery from echogenic liposomes to *ex vivo* perfused porcine

1125 carotid arteries induces potent vasorelaxation. The vasodialative effect of nitric oxide-loaded
1126 echogenic liposomes upon insonification (5.7 MHz, 0.36 MPa peak negative pressure, 30 s
1127 treatment) was also shown in *ex vivo* perfused rabbit carotid arteries with arterial wall
1128 penetration of nitric oxide confirmed by fluorescence microscopy (Kim, et al. 2014). In
1129 addition to this, vasodialative effects were demonstrated in carotid arteries *in vivo* in rats with
1130 vasospasms following subarachnoid hemorrhage using 1 MHz ultrasound with 0.3 MPa peak-
1131 to-peak pressure, 50% duty cycle for a duration of 40 min with constant infusion of the
1132 echogenic liposomes. This resulted in improved neurological function (limb placement, beam
1133 and grid walking) (Kim, et al. 2014). Ultrasound-activation of the antioxidant hydrogen gas
1134 encapsulated in microbubbles was shown to prevent myocardial ischemia-reperfusion injury in
1135 rats when administered before reperfusion (He, et al. 2017). There was a dose-dependent effect
1136 as 2×10^{10} microbubbles resulted in a more significant reduction in infarct size (70%) than 4
1137 $\times 10^9$ microbubbles (39%) compared to vehicle-treated rats. Furthermore, treatment with the
1138 high dose hydrogen-microbubbles prevented changes in left ventricular end-diastolic and left
1139 ventricular end-systolic dimension as well as minimal reductions in ejection fraction and
1140 fractional shortening. Histological and ELISA analysis showed a reduced degree of myocardial
1141 necrosis, apoptosis, hemorrhaging, inflammation, and oxidant damage. At the same time that
1142 cardiovascular drug delivery and therapy using microbubbles and ultrasound is moving
1143 forward to large animal and clinical studies, sophisticated *in vitro* models are being used and/or
1144 developed for mechanistic studies, such as flow chambers (μ Slides, Ibidi) (Shamout, et al.
1145 2015) and perfused 3D microvascular networks (Juang, et al. 2019) in which human umbilical
1146 vein endothelial cells are grown.

1147

1148 **Clinical study**

1149 Microbubbles and ultrasound were clinically investigated to augment muscle blood flow in
1150 12 patients with stable sickle cell disease in the absence of a drug at the Oregon Health &
1151 Science University, Portland, Oregon, USA (Belcik, et al. 2017). Perfusion increased ~2-fold
1152 in the forearm flexor muscles upon Definity[®] infusion and insonification at 1.3 MHz (MI 1.3).
1153 Ultrasound was applied 3 times for 3 min with ~5 min intervals. The change in perfusion was
1154 determined from contrast enhanced ultrasound imaging and extended well beyond the region
1155 where ultrasound was applied. This study showed that the therapeutic ultrasound settings
1156 directly translate from mouse to man for superficial muscles, as the same investigators
1157 demonstrated augmented blood flow in ischemic and non-ischemic hindlimb muscles in mice
1158 in the same study and an earlier publication (Belcik, et al. 2015). However, for the preclinical
1159 studies custom-made microbubbles were used instead of Definity[®].

1160

1161 **SONOBACTERICIDE**

1162 Sonobactericide has been defined as the use of ultrasound in the presence of cavitation
1163 nuclei for the enhancement of bactericidal action (Lattwein, et al. 2018). This topic has recently
1164 gained attention with 17 papers being published in the last five years. Research on ultrasound-
1165 mediated enhancement of antimicrobials has focused on several sources of infections including
1166 general medical devices (Ronan, et al. 2016, Dong, et al. 2017, Dong, et al. 2018, Hu, et al.
1167 2018, Fu, et al. 2019), acne (Liao, et al. 2017), chronic bacterial prostatitis (Yi, et al. 2016),
1168 infective endocarditis (Lattwein, et al. 2018), pneumonia (Sugiyama, et al. 2018), prosthetic
1169 joint infections (Li, et al. 2015, Lin, et al. 2015, Guo, et al. 2017a, Zhou, et al. 2018), or urinary
1170 tract infections (Horsley, et al. 2019). However, there was no specific disease aim in two studies
1171 (Zhu, et al. 2014, Goh, et al. 2015). One group targeted membrane biofouling for water and
1172 wastewater industries (Agarwal, et al. 2014). Direct bacterial killing, biofilm degradation and
1173 dispersal, and increased or synergistic therapeutic effectiveness of antimicrobials have been

1174 reported as the therapeutic effects of sonobactericide. These studies show that sonobactericide
1175 can be applied to treat Gram+ or Gram- bacteria, when they are planktonic, associated with a
1176 surface and embedded in biofilm, or intracellular. The majority of these studies were carried
1177 out *in vitro*. However, seven were performed *in vivo* in either mice (Li, et al. 2015, Liao, et al.
1178 2017, Sugiyama, et al. 2018, Zhou, et al. 2018), rats (Yi, et al. 2016), or rabbits (Lin, et al.
1179 2015, Dong, et al. 2018). Sonobactericide was mostly performed with co-administration of
1180 antimicrobials. Investigators also employed an antimicrobial encapsulated in liposomes that
1181 were conjugated to the microbubbles (Horsley, et al. 2019), or the antimicrobial lysozyme was
1182 a microbubble coating (Liao, et al. 2017), or did not use antimicrobials altogether (Agarwal, et
1183 al. 2014, Goh, et al. 2015, Yi, et al. 2016). An extensive review of sonobactericide has been
1184 published recently by Lattwein et al. (2019). Although sonobactericide is an emerging strategy
1185 to treat bacterial infections with intriguing potential, the mechanism and the safety of the
1186 treatment should be explored, particularly regarding biofilm degradation and dispersal. Future
1187 studies should also focus on maximizing the efficacy of sonobactericide *in situ*.

1188

1189 **FUTURE PERSPECTIVES AND CONCLUSIONS**

1190 Therapeutic ultrasound technology is experiencing a paradigm shift in terms of both
1191 technical developments and clinical applications. In addition to its inherent advantages for
1192 imaging (e.g., real time nature, portability and low cost), ultrasound in combination with
1193 cavitation nuclei is under exploration as a drug delivery modality. The results from several
1194 preclinical studies have already demonstrated the potential of ultrasound-responsive cavitation
1195 nuclei to deliver multiple types of drugs (including model drugs, anticancer, therapeutic
1196 antibodies, genes, nanoparticles, etc.) efficiently in various tumor models, including both
1197 ectopic and orthotopic models, for immunotherapy, brain disease, to promote the dissolution
1198 of clots, and in the treatment of cardiovascular disease and bacterial infections.

1199 Based on these encouraging preclinical data, several clinical trials have been initiated and
1200 others are planned. However, whilst animal studies provide proof of concept, and impetus for
1201 clinical studies, careful attention must be given to their relevance in human disease; in
1202 particular, the applicability of therapeutic protocols, and appropriate ultrasound settings.
1203 Otherwise we risk underestimating the therapeutic effects and potential deleterious side effects.
1204 The elucidation of all of the interactions between cavitation nuclei – cells and drugs will help
1205 to address this need. The biggest challenges lie in the large differences in timescales between
1206 the cavitation nuclei, drug release and uptake, and the biological response (Figure 8). A
1207 multidisciplinary approach is needed to tackle these challenges integrating expertise in physics,
1208 biophysics, biology, chemistry, and pharmacology.

1209 Custom-made microbubbles which serve as cavitation nuclei are often used for ultrasound-
1210 mediated drug delivery studies. An advantage is full control over the payload, as well as the
1211 disease target. At the same time, full acoustical characterization and sterility of the
1212 microbubbles must be considered during translation to human studies, which often requires
1213 approval from the United States Food and Drug Administration (FDA) or other similar federal
1214 agencies in Europe and Asia. As an example, for gene therapy, will each different type of
1215 genetic material loaded onto microbubbles need such approval, or will a class of cationic
1216 microbubbles be approved regardless of the specific gene? The former path would hinder fast
1217 clinical translation. For now, co-administration of drugs with FDA-approved ultrasound
1218 contrast agents is being explored in clinical trials. Apart from applications in the brain, ongoing
1219 clinical studies evaluating microbubble-mediated drug delivery are based on standard clinical
1220 ultrasound scanners operating mostly in Doppler mode. In order to promote the progress of this
1221 emerging technology, it is very important to design and implement specific therapeutic
1222 ultrasound pulse sequences that might be vastly different from clinical diagnostic imaging
1223 output. Clinical scanners can indeed be modified to be able to generate drug delivery protocols.

1224 In a similar way that elastography requires long ultrasound pulses to generate the push
1225 sequences (Deffieux, et al. 2009) , ultrasound scanners can be modified to be able to transmit
1226 drug delivery ultrasound sequences with tailored and optimized parameters (pulse duration,
1227 duty cycle, and center frequency).

1228 Ultimately, ultrasound image-guided drug delivery and the monitoring of treatment
1229 response could be feasible with the same equipment. Additionally, with recent developments
1230 in ultrasound imaging technology, ultrasound-mediated therapy could be planned, applied and
1231 monitored in a rapid sequence with high spatial and temporal resolution. The use of a single
1232 imaging and therapy device would alleviate the need for co-registration, because the imaging
1233 equipment would also be used to induce localized therapy ensuring a perfect co-location.
1234 Nonetheless, a compromise between efficacy and safety remains a major challenge for
1235 successful clinical applications of this dual methodology, which combines real-time image
1236 guidance of therapeutic delivery.

1237 In conclusion, ultrasound-responsive microbubbles which serve as cavitation nuclei are
1238 being used to treat a wide variety of diseases and show great potential preclinically and
1239 clinically. The elucidation of the cavitation nuclei – cell – interaction and the implementation
1240 of drug delivery ultrasound sequences on clinical ultrasound scanners are expected to
1241 invigorate clinical studies.

1242

1243 **ACKNOWLEDGEMENTS**

1244 Financial support from the European Research Council (ERC) under the European Union's
1245 Horizon 2020 research and innovation programme (grant agreement No 805308; PI: KK),
1246 Phospholipid Research Center in Heidelberg, Germany (PhD grant; PI: KK), FWO Vlaanderen
1247 (grant 12E3916N), U.S. Department of Health and Human Services, National Institutes of
1248 Health, National Institute of Neurological Disorders and Stroke grant R01 NS047603 (PI:

1249 CKH), Engineering and Physical Sciences Research Council (grants EP/I021795/1 and
1250 EP/L024012/1; PI: ES), the Canada Research Chair Program (PI: KH, PI: MAO) is gratefully
1251 acknowledged.

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2193

2194 **FIGURE CAPTIONS LIST**

2195 **Figure 1.** Combined effect of nonlinear propagation and focusing of the harmonics in a
2196 perfluoropentane micrometer-sized droplet. The emitted ultrasound wave has a frequency of
2197 3.5 MHz and a focus at 3.81 cm, and the radius of the droplet is 10 μm for ease of observation.
2198 The pressures are given on the axis of the droplet along the propagating direction of the
2199 ultrasound wave, and the shaded area indicates the location of the droplet (reprinted with
2200 permission from Sphak et al. (2014)).

2201

2202 **Figure 2.** Ultrasound-activated microbubbles can locally alter the tumor microenvironment
2203 through four mechanisms: enhanced permeability, improved contact, reduced hypoxia, and
2204 altered perfusion.

2205

2206 **Figure 3.** Schematic overview of how microbubbles and ultrasound have been shown to
2207 contribute to cancer immunotherapy. From left to right: microbubbles can be used as antigen
2208 carriers to stimulate antigen uptake by dendritic cells. Microbubbles and ultrasound can alter
2209 the permeability of tumors thereby increasing the intratumoral penetration of adoptively
2210 transferred immune cells or checkpoint inhibitors. Finally, exposing tissues to cavitating
2211 microbubbles can induce sterile inflammation by the local release of DAMPS.

2212

2213 **Figure 4.** 3D transcranial subharmonic microbubble imaging and treatment control *in vivo* in
2214 rabbit brain during BBB opening. Spectral information (top) shows the appearance of
2215 subharmonic activity at $t = 35$ s into the treatment. Passive mapping of the subharmonic band
2216 localizes this activity to the target region. Scale bar indicates 2.5 mm (reprinted (adapted) with
2217 permission from Jones et al. (2018)).

2218

2219 **Figure 5.** T₁ weighted sagittal MR images showing leptomeningeal tumors in rat spinal cord
2220 (grey arrowheads) before ultrasound and microbubble treatment (left column), and the
2221 enhancement of the cord indicating BSCB opening (white arrows) post-ultrasound and
2222 microbubble treatment (right column) (reprinted (adapted) with permission from O'Reilly et
2223 al. (2018)).

2224

2225 **Figure 6.** Simulated acoustic pressure and temperature in a representative subject exposed to
2226 pulsed 220 kHz ultrasound with a 33.3% duty cycle. The absolute peak-to-peak pressure
2227 maximum for the simulations is displayed in gray scale. Temperature is displayed using a heat
2228 map with a minimum color priority write threshold of 1 °C. Computed tomography features
2229 such as bone (cyan), skin and internal epithelium (beige), and clot (green), are plotted using
2230 contour lines. The transducer is outlined in magenta. Constructive interference is prominent in
2231 the soft tissue between the temporal bone and the transducer. Some constructive interference
2232 is also present in the brain tissue close to the contralateral temporal bone, however, the pressure
2233 in this region did not exceed the pressure in the M1 section of the middle cerebral artery.
2234 Temperature rise was prominent in the ipsilateral bone along the transducer axis.
2235 Computational model is described in Kleven et al. (2019).

2236

2237 **Figure 7.** Histological sections of a coronary artery of a pig 28 days after angioplasty. Pigs
2238 were treated with sirolimus-loaded microbubbles only (a) or sirolimus-loaded microbubbles
2239 and ultrasound (b) using a mechanically rotating intravascular ultrasound catheter (5 MHz, 500
2240 cycles, 50% duty cycle, 0.6 MPa peak negative pressure). Treatment with ultrasound and
2241 sirolimus-loaded microbubbles reduced neointimal formation by 50%. In both sections the
2242 intima (I) and media (M) are outlined; scale bar is 500 μm (Reprinted by permission from
2243 Springer Nature: Springer, Annals of Biomedical Engineering, Reducing Neointima
2244 Formation in a Swine Model with IVUS and Sirolimus Microbubbles, Kilroy JP, Dhanaliwala
2245 AH, Klibanov AL, Bowles DK, Wamhoff BR, Hossack JA, COPYRIGHT (2015)).

2246

2247 **Figure 8.** Different time scales of the therapeutic effects of ultrasound and cavitation nuclei
2248 treatment. $[Ca^{2+}]_i$ = intracellular calcium; ROS = reactive oxygen species; ATP = adenosine

2249 triphosphate; EV = extracellular vesicles (reprinted (adapted) with permission from Lattwein
2250 et al. (2019)).















