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Review article

# Safety and efficacy of focused ultrasound induced blood-brain barrier opening, an integrative review of animal and human studies

Ying Meng[a,b], Christopher B. Pople[b], Harriet Lea-Banks[b], Agessandro Abrahao[b,c], Benjamin Davidson[a,b], Suganth Suppiah[a], Laura M. Vecchio[b], Nardin Samuel[a], Faiza Mahmud[b], Kullervo Hynynen[b,d], Clement Hamani[a,b,e], Nir Lipsman[a,b,e,*]

[a] Division of Neurosurgery, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Canada
[b] Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Canada
[c] Department of Medicine (Neurology), Sunnybrook Health Sciences Centre and University of Toronto, Toronto, Canada
[d] Department of Medical Biophysics, University of Toronto, Toronto, Canada
[e] Hurvitz Brain Sciences Research Program, Harquail Centre for Neuromodulation, Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, University of Toronto, Toronto, Canada

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ABSTRACT

The blood-brain barrier, while fundamental in maintaining homeostasis in the central nervous system, is a bottleneck to achieving efficacy for numerous therapeutics. Improved brain penetration is also desirable for reduced dose, cost, and systemic side effects. Transient disruption of the blood-brain barrier with focused ultrasound (FUS) can facilitate drug delivery noninvasively with precise spatial and temporal specificity. FUS technology is transcranial and effective without further drug modifications, key advantages that will accelerate adoption and translation of existing therapeutic pipelines. In this review, we performed a comprehensive literature search to build a database and provide a synthesis of ultrasound parameters and drug characteristics that influence the safety and efficacy profile of FUS to enhance drug delivery.

## 1. Introduction

The blood-brain barrier (BBB) is a selectively permeable border between the systemic circulation and parenchyma of the central nervous system (CNS) [1]. The barrier is comprised of tight junctions between endothelial cells on the vessel wall that limit paracellular transport. In addition, the BBB consists of various efflux proteins, including ATP-binding cassette transporters, such as P-glycoproteins (P-gp) and solute carrier family transporters, that remove compounds from the CNS via active transcellular transport [2]. In simplified terms, the BBB is permeable to ions (i.e. $O_2$, $CO_2$) and small lipid-soluble molecules less than 400 Daltons (Da) [3]. The BBB is evolutionarily conserved in all vertebrates, being critically important for maintaining the brain microenvironment through regulating the composition of the cerebrospinal and interstitial fluid, peripheral and central cellular signaling, and immunity [4,5]. Notably, the subtle to overt alterations in BBB integrity noted in various disease pathologies such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis, are an area of active investigation [6].

While the BBB is fundamental in maintaining homeostasis and preventing the entry of potentially harmful compounds such as toxins and inflammatory proteins, it presents a bottleneck to achieving therapeutic efficacy for many drugs in the brain. Improving brain penetration may also be beneficial for reducing dose, associated cost, and side effects. Approximately 5% of more than 7000 drugs in the Comprehensive Medicinal Chemistry database are indicated for the CNS. Therefore, ensuring sufficient CNS penetration, such as by optimizing distribution kinetics or modulation of BBB permeability, should be a key consideration in developing any therapeutics for neurological disorders.

The modes of enhancing delivery include (in order of increasing invasiveness): co-administration of compounds that increase BBB permeability or inhibit efflux transporters (e.g. mannitol, elacridar) [7]; re-engineered drugs to hijack surface receptors for transcellular transport (e.g. transferrin receptor) [8]; radiation therapy [9] to disrupt the BBB; and open surgery for direct injection (e.g. convection-enhanced delivery CED) (Fig. 1). As an example, valanafusp alpha, a systemically administered insulin receptor antibody-iduronidase fusion protein, was

* Corresponding author at: Harquail Centre for Neuromodulation, Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, Division of Neurosurgery, 2075 Bayview Avenue, Toronto, Canada.
*E-mail address:* nir.lipsman@sunnybrook.ca (N. Lipsman).

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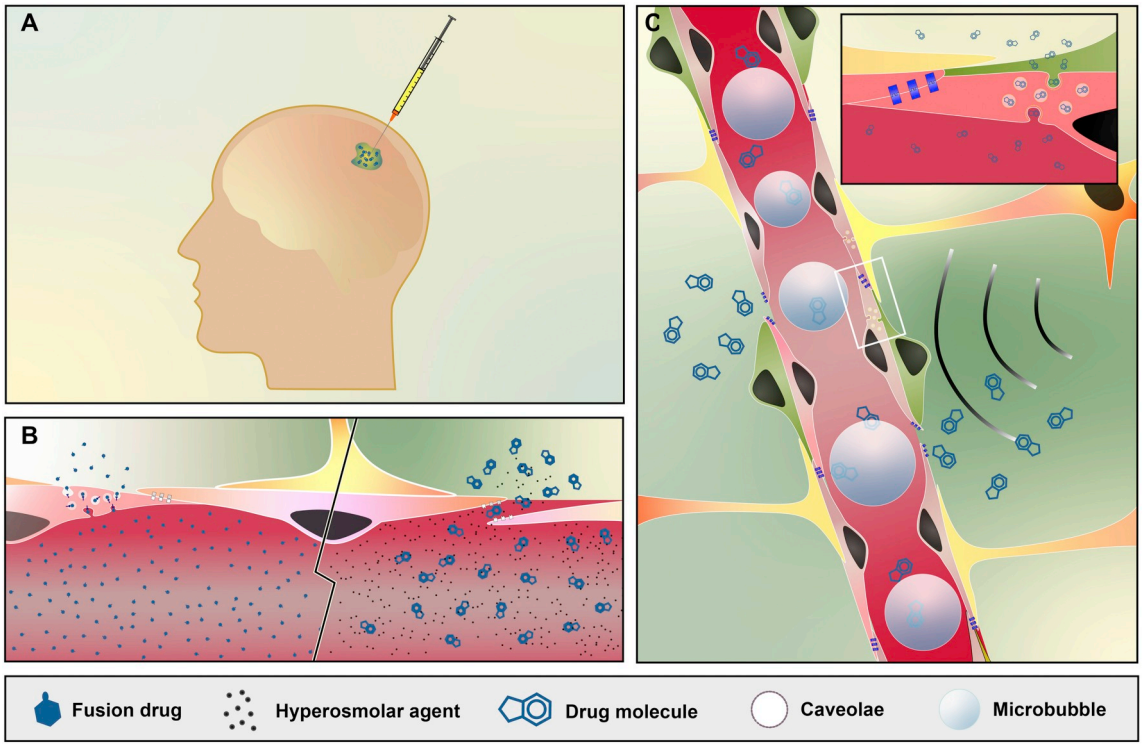


**Fig. 1.** Schematic diagram illustrating technologies available to enhance drug penetration past the BBB. (A) Convection-enhanced delivery involves controlled direct injection of the drug into the brain parenchyma, once or over the long-term via an implanted pump. (B) Left panel shows re-engineered drugs that are fused to insulin or transferring receptors to pass through the BBB via transcellular transport. Right panel shows disruption of the BBB by co-administration of hyperosmolar agent (e.g. mannitol). (C) FUS and microbubbles enhance drug penetration through the BBB primarily by mechanical disruption of tight junctions, but also via increased caveolae-mediated transport (inset). Expression of P-glycoproteins, not shown, is also downregulated after treatment.

successfully tested for severe Mucopolysaccharidosis Type I in a phase 1-2 trial [10]. CED is commonly used by surgeons to introduce a large amount of drug to a localized region. Recent clinical trials employing CED include the introduction of chemotherapy for diffuse intrinsic pontine glioma and neurotrophic factor for Parkinson's disease (PD) [11,12]. In contrast, focused ultrasound (FUS) with intravenous microbubbles (MB) is an emerging approach to increasing CNS penetration that is noninvasive; sonication of intravascular MBs within a brain target temporarily increases local BBB permeability, creating a window of opportunity for drug delivery.

There are several unique aspects to FUS technology. First, compared to re-engineered drugs targeting transferrin receptors or systemic hyperosmolar agents, FUS is spatially precise and obviates further drug modifications, which might be time-intensive and costly. Second, compared to invasive CED, FUS is an incision-less approach to drug delivery without foreign body implants, which is more attractive for patients. Furthermore, FUS can aid drug delivery to focal yet widely distributed target brain regions. Finally, a variety of parameters, such as ultrasound and MB dosing, can theoretically be fine-tuned to achieve an appropriate level of BBB opening.

FUS has been studied in numerous animal models of disease, where the technology has shown great promise in enhancing therapeutic bioavailability. As FUS is poised to move forward into clinical trials, the first question concerns safety. Namely, what are the short-term and long-term histological, biochemical, and behavioral consequences of increasing BBB permeability – once and over the course of multiple applications? The next question is efficacy: to what degree does FUS BBB opening increase drug concentrations in the CNS? In order to address these questions, we performed a systematic review of the literature to build a database and synthesize experimental conditions and drug characteristics that influence the safety and efficacy profile of FUS to enhance drug delivery.

## 2. Method of literature search

We searched PubMed in October 2018 for all studies on FUS in conjunction with MB induced BBB disruption using search terms (focused ultrasound OR FUS) OR (MRgFUS OR MR-guided focused ultrasound OR MRIgFUS) AND (BBB OR blood-brain barrier). Two authors screened the titles and abstracts with pre-determined exclusion criteria. We excluded articles that were simulation or *ex vivo* studies, not in English, did not have data directly relevant to safety or did not use low-intensity FUS or MB. We then proceeded with full-text screening followed by data extraction, which were performed using a standardized form (Table S1). We collected information regarding the animal model (i.e. species), anatomic target, primary objective of the study, ultrasound parameters, timing and technique of safety characterization, as well as effect on drug delivered if available. Data was then summarized qualitatively and graphically.

## 3. Overview of existing studies

Our search yielded 422 studies, from which 165 studies were included for data extraction and synthesis (Fig. S1). Since the first descriptions of FUS and MB induced BBB disruption in rabbits, research activity in this field has dramatically increased with publications distributed mainly in specialized journals and some clinical journals (Fig. 2a) [13]. While all included studies addressed safety, the objectives of sixty-four (38%) were primarily to establish the optimal FUS parameters and conditions for safe and effective BBB opening. Five studies focused on the effect of FUS on neural activity with potential implications for the development of FUS for neuromodulation.

FUS-induced BBB opening has been investigated in multiple species with a wide range of skull volumes and thickness: prominently small rodent models with fewer studies in large animal models (e.g. swine,

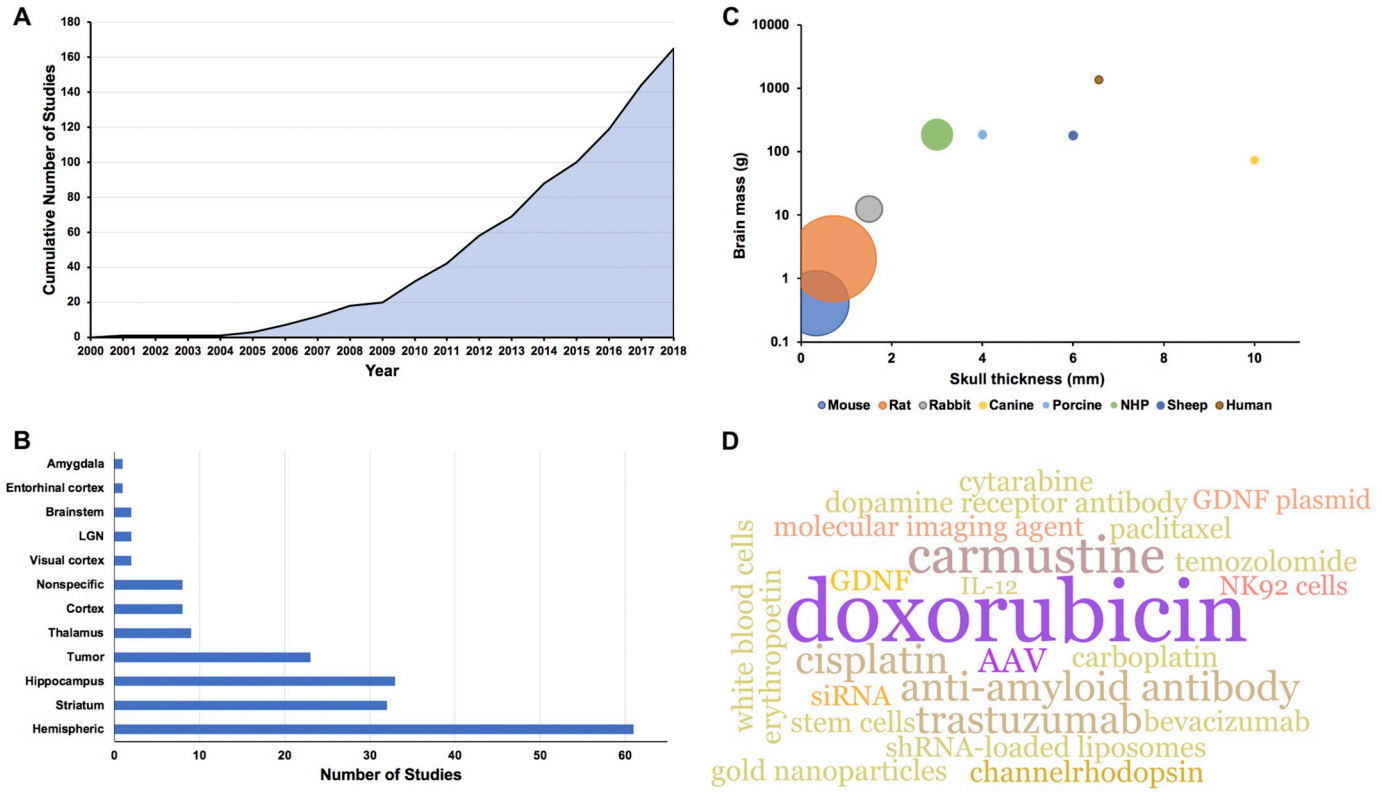


**Fig. 2.** Summary of representative trends and statistics relevant to the included publications. (a) Cumulative publications by year. (b) Plot of animal model by skull thickness vs brain mass, with the size of the circle proportional to the number of publications. (c) Number of studies targeting particular region for BBB opening. (d) A word cloud of various drugs and deliverables by FUS, with font size proportional to number of studies.

canine, sheep, nonhuman primates (NHP)) and human subjects with targets in hippocampus, striatum, thalamus, brainstem and tumor tissue (Fig. 2b,c). Nearly all experimental set-ups employ a single transducer, as opposed to the multi-phased array device used in clinical trials. For small animals, a single transducer will cover a significant volume of tissue, accompanied by relatively low spatial specificity, often resulting in hemispheric BBB disruption. More flexible target configurations can be achieved with phased arrays with varying patterns of transducer alignment and activated elements.

Sixty-nine (42%) studies explore the potential of FUS to deliver therapeutic compounds relevant for neuro-oncology (43), Alzheimer's disease (9), Parkinson's (18), other neurodegenerative disorders (3), traumatic brain injury (1), stroke (1), and epilepsy (1) (Fig. 2d). In these investigations, both healthy and diseased brains have been tested, including but not limited to amyloid and tau models for AD, 6-OHDA and alpha-synuclein models for PD, glioma and brain metastasis models.

## 4. Safety

Potential deleterious effects of FUS-induced BBB opening include microhemorrhage, overt hemorrhage from vascular rupture, ischemia from vascular constriction, cerebral edema and/or inflammation from protein extravasation, as well as direct cellular injury from heat or mechanical forces.

Iron deposition is another potential contributor to brain toxicity after major intracranial hemorrhage [14]. Its consequences after FUS are unknown but are likely compensated by neuroglia in the case of microhemorrhages. Assessments of safety can be made at the macroscopic, microscopic, and biochemical levels through in vivo imaging techniques (e.g. magnetic resonance imaging MRI), behavioral testing, histology, and biomolecular assays at acute and delayed timepoints. While the two latter techniques have greater sensitivity, the previous ones permit repeated, longitudinal measurements in live animals or human subjects. For instance, hemorrhage and tissue damage can be scored as grade 0 - normal tissue, 1 - scattered or discontinuous erythrocyte extravasation, 2 - continuous extravasation or microhemorrhage, and 3 - hemorrhage with necrotic damage or gross hemorrhage (Fig. 3) [15]. Microhemorrhages can be detected via T2* or susceptibility-weighted (SWI) MRI sequences, but these sequences lack the ability to detect grade 1 hemorrhage [16]. The presence of necrotic cells, apoptotic cells, or vacuolations can also be assessed via hematoxylin & eosin (H&E) histology and other markers such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL).

While most studies employ similar methodology to characterize safety, a statistical summary remains difficult due to the wide variability in experimental set-ups (e.g. ultrasound parameters, MB type and dosage), and animal models. In fact, the reporting of experimental settings (e.g. ultrasound parameters, method of pressure estimation, microbubble type and dose) was complete only for 70% of the studies. Here, we synthesize the literature to summarize the safety profile of FUS.

### 4.1. Thermal effects

FUS-induced BBB opening uses low-intensity ultrasound, therefore is expected to have minimal thermal effects. On the other hand, high-intensity FUS, used for thermoablation, deposits heat in biological tissue, causing noninvasive, rapid thermocoagulative necrosis at temperatures in excess of 55°C [17]. Typical powers necessary for thermoablation are in the range of 300-800 W 20-30s when sonications are performed with a hemispherical array operating at the frequency of about 650kHz. FUS-induced BBB opening is achievable with a time-averaged power more than three orders of magnitude lower due to the

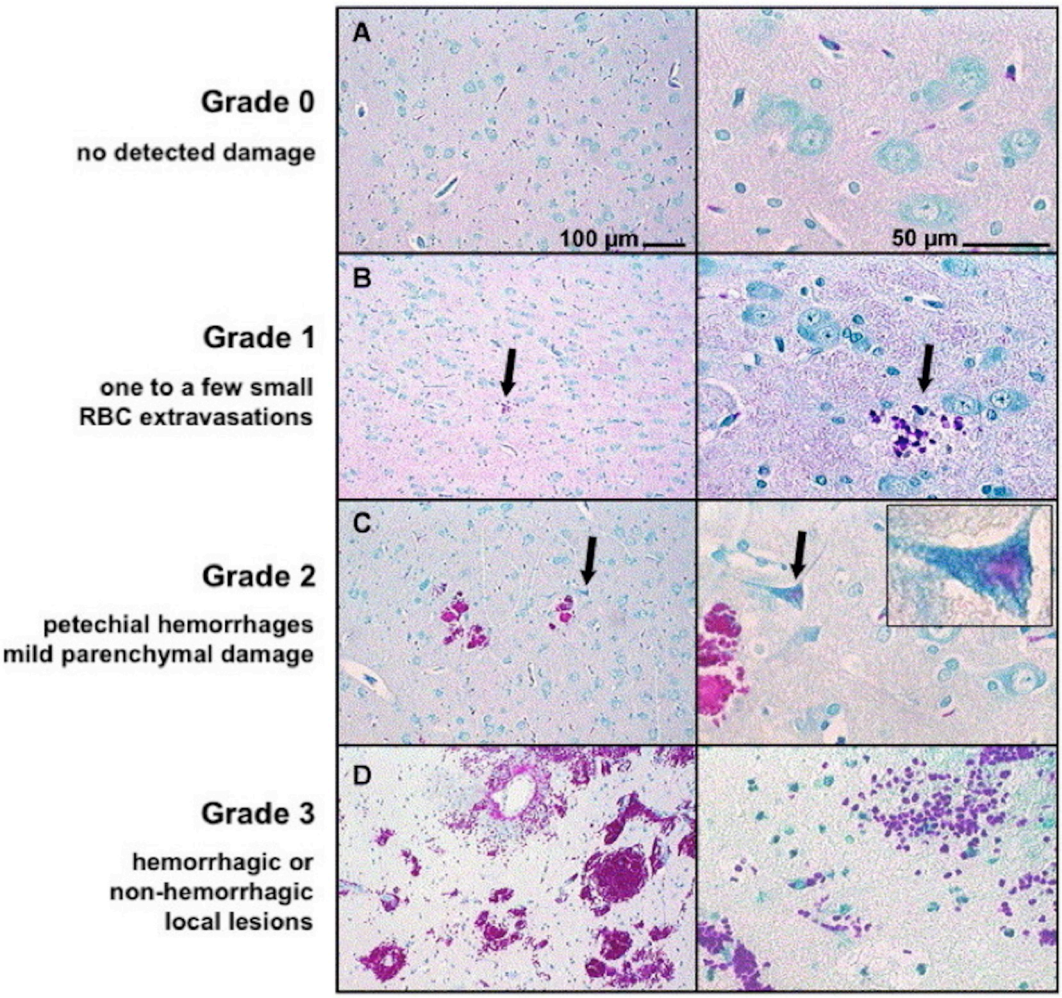


**Fig. 3.** Examples of histological VAF–toluidine sections representative of classification commonly used to grade hemorrhages in FUS for BBB opening studies. (A) Grade 0: No observable damage. (B) Grade 1: one to a few RBC extravasations within the focal region. (C) Grade 2: Scattered petechiae consisting of microscopic areas of perivascular extravasations within the sonicated area, potentially some damage to the parenchyma (magnified insert shows an ischemic cell). (D) Grade 3: Extensive RBC extravasation, potentially accompanied by a region of infarction. Adapted with permission from Hynynen et al. *NeuroImage* 2005.

exogenous introduction of ultrasound contrast agents, microbubbles, that create nucleation for mechanical effects thought to underlie BBB disruption [18]. Negligible temperature elevations ($< 3^{o}C$) were confirmed in three studies with different ultrasound parameters in rat, rabbit, and sheep [19–21].

### 4.2. Mechanical effects

The target tissue is at a higher risk of injury due to mechanical rather than thermal effects from the interactions between FUS and microbubbles. FUS-induced BBB opening is thought to occur via mechanical stretching, acoustic streaming, and shear forces from MB oscillations on the vasculature [22]. These forces can affect the function and structure of tight junctions and the expression of transporter proteins such as P-gp, as well as increase endocytic vesicles in the BBB [23,24]. The degree of BBB opening is thought to be graduated. In other words, higher tissue drug concentrations or successful penetration of higher molecular weight drugs can be found with incremental levels of BBB opening. For instance, at safe FUS-induced BBB opening levels, tissue penetration of 2000 kDa dextran was negligible [25]. Therefore, achieving a level of BBB opening to allow sufficient therapeutic access must be balanced with the risks of vessel injury with higher mechanical effects. In some instances, the therapeutic window may be small, particularly when applying to diseased brains with a low level of acceptable risk.

Early studies show the transducer frequency, ultrasound intensity and microbubble size and dosage can affect the extent of BBB disruption and its safety profile [26]. At 1.5 MHz, grade 3 hemorrhage with apoptosis and tissue damage was detected with ultrasound pressure of 2.4 MPa at 690 kHz, and grade 2 with 1.6 MPa [27,28]. At 1 MHz, in a rat neuro-oncological model, apoptosis and grade 2 hemorrhage resulted from pressures greater than 1.5 MPa, and grade 1 from pressures between 0.3 - 0.5 MPa [29]. In NHPs, evidence suggests 0.185-0.266 MPa is a safe range for the striatum with a 690 kHz transducer [30]. Limited and occasional T2* changes on MRI did not correspond to any clinical change or delayed BBB closure.

Subsequent optimization studies showed that microhemorrhages were avoidable whilst still achieving BBB opening at lower sonication powers. The BBB was more readily disrupted at lower pressures with lower transducer frequencies [29]. Consequently, the mechanical index (MI) was used to standardize and help interpret the heterogeneity between experimental setups. The MI is the peak negative pressure amplitude estimated *in situ* divided by the square root of the ultrasound frequency, and appears to be constant for the threshold (50% probability) for BBB disruption [31]. Grade 3 hemorrhage accompanied MI of 1.5, grade 1-2 hemorrhage with MI > 0.5, and none with MI of 0.45 [31,32]. Potential delayed or more subtle adverse events of FUS BBB opening on tissue include ischemia from vasoconstriction, atrophy, formation of cystic cavities, and mineralization [33–36], while other

studies did not find such delayed structural changes with MI of 0.49, or after repetitive FUS-induced BBB opening [37].

MBs are nucleation sites for the mechanical effects of FUS and play an essential role in the safety profile of FUS. Several studies are dedicated to characterizing the impact of MB characteristics and dosage. Three commercial formulations of ultrasound contrast agents are often used: Definity, Optison, and SonoVue, at 1-3 um, 2-4 um, and 2-5 um in size respectively [38–40]. Different MB size or coating, such as with a drug, can alter the MB resonance frequency and behavior under ultrasound exposure. Furthermore, larger MBs (e.g. SonoVue) or higher dosage with longer effective circulation time are associated with a higher risk of hemorrhage given the same ultrasound parameters [41–46]. At the same time, larger-sized MBs, at a fixed concentration, were found to have a longer circulation time [47]. Choi et al. showed in a healthy mouse model, with a 1.525 MHz transducer, the threshold for BBB opening was between 0.30-0.46 MPa for 1-2 μm MBs, and 0.15-0.30 MPa for 4-5 μm MBs [48]. A unifying parameter combining MB size and concentration was also proposed, but the dependence of bubble oscillations for BBB-opening on the bubble size and ultrasound frequency remains to be tested in other studies [49].

Efforts to create a universal language are important to improved safety, efficacy, and reproducibility of results, and eventually for moving towards clinical translation. Furthermore, recent studies increasingly employ real-time acoustic feedback controllers with passive cavitation detection to optimize safety, which we expect to see more widespread implementation of in both preclinical and clinical devices [30,50–52].

### 4.3. Risk of inflammation

Inflammation is a natural response to injury or stress to restore and maintain homeostasis. The role of inflammation in the CNS is multifaceted; it may aid recovery on the one hand and exacerbate pathology on the other. An inflammatory response can be broadly classified as acute and chronic. While acute inflammation is restorative after the initial injury, chronic inflammation is generally considered detrimental [53]. The BBB is vital in maintaining the microenvironment by restricting the passage of harmful and pro-inflammatory substances such as albumin and fibrinogen from the systemic circulation. The presence of fibrinogen, for instance, has been shown to inhibit remyelination and repair in neuropathology [54].

Indeed, extravasations of circulating albumin and endogenous immunoglobulins (e.g. IgG and IgM)have been demonstrated after FUS BBB opening [35,55]. An increase in parenchymal albumin along with increased cytokines and cell adhesion molecules, consistent with a sterile inflammatory response, was observed in one study [35]. While the response lasted no more than twenty-four hours, these changes are concerning for their impact on normal vascular and neurovascular unit physiology. An acute inflammatory response is further supported by transcriptomic changes (e.g. IL-6, TNF-alpha) as well as microglia and astrocyte activation, albeit the latter was self-resolving in approximately two weeks [56]. On the other hand, activated microglia, thought to be triggered by leaked endogenous immunoglobulins, have been found to cluster around amyloid plaques, in a process that potentially facilitates amyloid clearance in a transgenic model of AD [57]. Therefore, whether acute inflammation post-FUS BBB opening is deleterious or beneficial is debated. Transcriptome analysis showed enhanced activation of the Akt/GSK3β pathway typically associated with survival [35,58]. In keeping with this, FUS BBB opening may be beneficial in modulating the microenvironment into a more permissive state in diseases where neuroinflammation is implicated in the pathophysiology. Another speculation is that FUS BBB opening facilitates immune recognition, or angiogenesis [59,60].

Larger substances, such as white blood cells, do not appear to penetrate the BBB with typical FUS BBB opening parameters but have been observed in conjunction with cases of microhemorrhage [59,61]. In certain situations, breaking immune-privilege might be acceptable or desirable, such as in the combined application of FUS and IL-12 to encourage an immune response to tumor antigens hidden behind the BBB [59]. Nevertheless, platelets and white blood cells, even without transmigration, could contribute to inflammation through activation, adherence and interaction with the vessel endothelium. Our understanding of the *in vivo* impact of FUS and microbubbles on these more complex processes and membrane dynamics specifically is relatively limited to early electron microscopy studies (e.g. increased visualization of vesicles, fenestrations and channel formations) [23]. A more detailed assessment of the changes in endothelial cell membranes and their interaction with circulating substrates would provide additional insight on FUS and inflammation.

The transition of an acute inflammatory microenvironment to a chronic one would be concerning given persistent neuroinflammation is thought to contribute to neurodegeneration. In one study, six weekly FUS exposure led to tissue atrophy with associated ventriculomegaly, cavity formation, mineralization, as well as increased phosphorylated tau [36]. However, data from a number of different laboratories show post-FUS the acute inflammatory mRNA profile is self-limited [51,56], microglial activation resolves by approximately two weeks and does not progress to chronic inflammation [55,62]. Glial scar or astrogliosis has not been evident on short- and long-term (e.g. months) examinations after singular or repeated FUS exposures [55,63–65]. Furthermore, no behavioral, neuroimaging, or morphologic changes were documented after biweekly BBB opening over four months in NHPs or six months in rats [66,67].

Differences in FUS exposures, bubble dose and other experimental settings may explain the discrepancies in adverse events. As previously discussed, the safety window is sensitive to a multitude of these factors. Indeed, BBB disruption induced by supra-threshold ultrasound will result in hemorrhage and glial scarring [28]. Furthermore, a weekly paradigm may be too closely spaced for recovery from acute inflammation, culminating in additive effects or chronic inflammation. Certainly, endeavoring to use FUS for the delivery of therapeutics with known immune-related adverse events(e.g. immunotherapies, gene therapies) deserves careful consideration of the potential additive risks [68,69]. Examples of this include autoimmune encephalitis from checkpoint inhibitors and amyloid-related imaging abnormalities (ARIA) from anti-amyloid antibodies. This is an important aspect of FUS safety that warrants further investigations.

### 4.4. Functional effects

Safety can be further assessed by behavioral testing before and after FUS application. Such tests might include motor (e.g. rotarod), visual (e.g. computerized visual response task), and learning (e.g. Y-maze) tasks. These results provide key information about the potential clinical relevance of biomolecular and histological changes. Additionally, electroencephalographic monitoring of well-characterized event-related potentials may be employed to assess neural correlates of the aforementioned outcomes and provide orthogonal measures of functional outcomes with a neurobiological basis. Similarly, high-dimensional data from functional MRI and metabolic PET imaging (i.e. $^{18}$F-FDG metabolism) may identify potentially relevant functional changes in brain activity. Taken together, such diverse measures of functional outcomes provide a broad, integrative understanding of the neurobiological alterations from FUS-induced BBB opening.

Several functional neuroimaging studies examining metabolism and blood-oxygen-level-dependent (BOLD) responses following treatment have been carried out in rats and NHPs. Functional MRI studies have consistently identified changes following BBB opening, including reduced BOLD responses and functional connectivity with sonicated regions [70,71]. Such results may signify true functional outcomes or reflect changes in neurovascular coupling and local blood flow, which have been observed following FUS-induced BBB opening in other

studies [33,34,70–74]. In fact, these changes resolved in hours to days depending on the intensity of FUS, with changes only persisting to 7 days when histological damage was observed [70,71]. $^{18}$F-FDG-PET scans to monitor *in vivo* glucose metabolism have yielded less consistent results. Work in an NHP model using an implantable ultrasound device showed no significant changes in uptake, whereas transcranial FUS in a rat model resulted in reduced uptake concomitant with suppression of glucose transporter expression [67,75]. Similarly, somatosensory evoked potential (SSEP) monitoring following FUS has revealed inconsistent results, with no changes in SSEPs in an NHP model employing an implanted device, but transient decreases in SSEP magnitude (but not latency) following single or repeated treatments in a rat model [67,70]. Notably, SSEP changes were correlated with the intensity of FUS, where higher intensities produced persistently reduced amplitude and increased latency, as well as histological damage and changes in BOLD response[70].

Although neuroimaging and electrophysiological measures provide highly granular, quantitative data about neurobiological processes, behavioral measures are the ultimate endpoints for potential dysfunction. Several studies included in this review employed behavioral endpoints for NHP models, frequently following or during repeated sonications over several weeks or months. Two other studies examined behavior in mice and rats after exposure to FUS at mild or more intense parameters. Notably, FUS-induced BBB opening in the brainstem of rats did not correspond to abnormalities in vitals monitoring or decreased rotarod performance [76]. Of those studies in NHPs, all found a complete absence of dysfunction in visual, motor, or social, or reward-motivated behaviours, despite multiple applications in numerous regions including the striatum, the thalamus, hippocampus, white matter tracts, and the visual, cingulate, or motor cortices with both implantable and transcranial systems. Such results were consistent even in the presence of identifiable damage in histological sections or by MRI assessment (e.g. T2*-weighted images) [30,67,77–79]. One study found improved performance in a visuomotor task following BBB disruption in the form of decreased reaction time and increased touch accuracy [78]. Work in murine models identified some alterations with decreased center entries on one testing session 18 days post-sonication and reduced body weight by the end of the study period in rats exposed to mild FUS [80]. Nonetheless, neither changes in grip strength nor memory were evident in these rats, nor were any changes observed in gross behavior, distance travelled, time spent moving, rotarod performance, open field test behavior, or turning in mice receiving mild unilateral FUS. However, more intense parameters induced significant impairments in motor behavior and memory spanning several tests, demonstrating the potential for harm with suboptimal parameters [66,80].

An increased number of cells co-labelled with bromodeoxyuridine (BrdU) and doublecortin (DCX), interpreted as increased neurogenesis, has been described in the target area of both healthy and transgenic AD mice approximately three weeks after FUS-induced BBB opening [57,63]. The underlying mechanism of this finding is unclear but has since been shown to be dependent on BBB opening [81]. Potential explanations or contributors include entry of stimulatory growth factors from increased BBB permeability, increased angiogenesis, activation of the pro-survival and proliferative Akt pathway, which is an important regulator of proliferation and migration of neural stem cells in other models such post-ischemia or trauma [35,58,60,82].

### 4.5. Clinical studies

Our search yielded one pilot clinical study that examined the safety and feasibility of FUS BBB opening in patients with mild-to-moderate AD [83]. This investigation employed ultrasound contrast agent Definity® and commercial MRgFUS device (InSightec ExAblate Neuro), which consists of an array of over 1000 transducers arranged within a dome. The study showed that the procedure was well-tolerated in five subjects with AD. A volume in the right frontal lobe was targeted twice over one month, all resulting in BBB closure in twenty-four hours. Subjects were followed up for three months without any clinically significant adverse events. One subject had a subtle T2* signal loss after BBB opening that resolved within a short period of twenty-four hours.

One ultrasound-induced BBB opening study in subjects with recurrent glioblastoma was not included in our search result because the trial used unfocused, pulsed ultrasound from a skull-implanted transducer system, CarThera® SonoCloud [84]. The tissue in line with the transducer was sonicated at fixed pressures with a concurrent injection of SonoVue®. Treatments were performed monthly in seventeen subjects without any adverse events detected clinically or by MRI (e.g. edema, ischemia, hemorrhage). Notably, because there was no acoustic feedback, ultrasound power was incrementally increased on each occasion to test the success of BBB opening. One subject suffered a cerebellar stroke, detected on immediate post-sonication MRI, that was thought to be unrelated to the procedure based on the spatial separation from the expected transducer beam. A larger study for patients with recurrent glioblastoma is ongoing (NCT03744026). Finally, another clinical device for BBB opening entering phase I trials is NaviFUS (NCT03626896), which like the ExAblate Neuro utilizes a phased array of transducers. In contrast, the NaviFUS eliminates the stereotaxy in favor of a frame-less neuro-navigation system.

### 4.6. Summary

A window of safe BBB opening via FUS exists, but it dictates the degree and characteristics of therapeutics (e.g. molecular weight) that might subsequently pass through. It will be essential to recognize the different priorities and trade-offs between safety and efficacy for different disease models. For instance, in certain cases, achieving successful drug delivery will outweigh minor, transient complications. FUS-induced BBB opening would not be suitable for other situations, particularly neurodegenerative disorders with significant inflammatory etiology (e.g. multiple sclerosis). Chronic administrations might be detrimental and further exacerbate pathology.

Finally, standardization of FUS procedures will be critical to effective communication between researchers, thereby enhancing safety and clinical adoption of this technology. This process is currently hampered by 1) the large parameter space that impacts BBB opening results, and 2) the challenge of accurately estimating ultrasound pressure *in vivo* due to skull variations amongst subjects. Still, efforts to do so include the development of composite measures (e.g. MI) and employing feedback systems (e.g. active or passive cavitation detection). Acoustic monitoring of the microbubble activity is perhaps the most promising approach to dosing ultrasound given different spatial locations, ultrasound parameters and instrumentation (e.g. transducer properties, design and placement). Currently, in clinical studies, the optimal ultrasound power for BBB opening is determined via a ramp test. The ramp test involves applying short sonications of incremental power until the cavitation threshold is detected on acoustic feedback from the target [50].

## 5. Efficacy

Successful delivery of a candidate drug by FUS-induced BBB opening can be quantified in a variety of ways, both directly and indirectly. *In vivo* imaging can quantify drugs directly via radiolabelled ligands (e.g. PET imaging) or indirectly via magnetic nanoparticles or conjugation of superparamagnetic iron oxide to microbubbles or cells (e.g. MRI). Although not amenable to clinical research, drug delivery can also be directly quantified by high-performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA) analysis of brain tissue homogenates, or indirectly by imaging of fluorescently-labelled delivery vectors such as nanoparticles. Similarly, delivery of gene therapy by vectors such as adeno-associated virus or liposomal plasmids can be assessed by imaging of fluorescent transgene

**Table 1**
Relative changes in therapeutic delivery via FUS-induced BBB opening

| Drug | Results |
|---|---|
| Dextran | 3 kDa: 30-82% signal coverage on histology [25]<br>70 kDa: 17-25% signal coverage on histology [25]<br>500 kDa: 8% signal coverage on histology [25]<br>2000 kDa: negligible [25] |
| Temozolomide (194 Da) | 1.7x increase in CSF-to-plasma ratio [105]<br>2.6x increase in tissue concentration, 1.6x increase in half life [106] |
| Cytarabine (243 Da) | 1.7-4.4x increase in tissue concentration [107,108]<br>similar tissue concentrations achieved between 4 mg/kg dose plus FUS and 50mg/kg dose without FUS [107] |
| BCNU (214 Da) | 2.02-5.69x increase in drug concentration vs control [39,109]<br>4.0-8.2x increase in drug concentration with FUS aided delivery BCNU MBs (0.86-1.32 μm), and up to 5x increase in half-life [29,109,110] |
| Doxorubicin (543 Da) | 2-16x increase in drug concentration [38,41,52,111–116]<br>56x increase in drug concentration [76] |
| Plasmid gene therapy | 1.4-2x increase in GDNF expression when GDNF-plasmid delivered with FUS vs control [104,117,118]<br>7x increase in transfection rate of FUS delivered DNA BNPs (56-65 nm in size) [99]<br>4.7x increase luciferase expression with plasmid-microbubble (3.2 μm) than direct injection [119]<br>20x increase in BDNF expression with BDNF plasmid-microbubbles (3.60 μm) delivered by FUS [120] |
| Imaging agents | 1.7x increase PET signal with FUS aided delivery of 18F-FBPA-Fr [121]<br>4x increase SUVR (at 45 min) with FUS aided delivery of hydrophilic molecular imaging agent (441 Da) [122]<br>1.5x increase SPECT signal in normal brain, 1.3x increase in tumor tissue with FUS aided delivery of 99mTc-DTPA (487 Da) [123]<br>3.8x increase in signal with FUS aided 123I-FIAU (372 Da) [124] |
| GDNF-microbubbles (24 kDa) | 16x increase concentration [125] |
| IL-12 (70 kDa) | 1.7x increase concentration [59] |
| Antibodies (150 kDa) | 5.5x increase in tissue concentration of bevacizumab with FUS at 0.4 MPa, 47x increase with 0.8 MPa [101]<br>1504 ng/g tissue trastuzumab concentration at 0.6 MPa, 3257 ng/g at 0.8 MPa with 20mg/kg injection, versus undetectable in control [26]<br>1.5-1.8x increase in endogenous IgG [55]<br>4.6-6.7x increase in endogenous IgM (900 kDa) [55] |
| AAV gene therapy (20nm, 4000 kDa) | 3-6.5x increase in GFP expression vs control [126–128]<br>GFP expressed in 74% of cells in FUS group [65] |
| shRNA-liposomes (241.4 nm) | 20-35% in transfection efficiency vs negligible [129] |
| HSV-TK/GCV-loaded VEGFR2 targeted MBs (1.1 μm) | 2x transgene expression [130] |
| Drug conjugated nanoparticles | 6.4-30x greater spatial tumoral coverage and up to 75x greater peritumoral coverage with FUS aided delivery of cisplatin NPs (60 nm) [131]<br>1.4-6.9x increase with FUS aided delivery of paclitaxel NPs (170nm) [132]<br>2x increase with FUS aided delivery of BCNU NPs (10-20nm) [133] |
| Erythropoetin (21-37 kDa) | 46.9x increase concentration in brain tissue, 2.7x increase in CSF with FUS [134] |
| Cells | 10x increase in number of NK-92 cells per tumor cell when delivered with FUS vs control [135]<br>15% signal change on MRI for SPIO-labelled WBCs [136]<br>32 $GFP^+$ cells/$mm^2$ vs negligible with FUS delivered stem cells [137] |

AAV: adeno-associated virus; BCNU: carmustine; BDNF: brain-derived neurotrophic factor; BNP: brain-penetrating nanoparticle; CSF: cerebrospinal fluid; GDNF: glial-derived neurotrophic factor; GFP: green fluorescent protein; HSV-TK/GCV-loaded VEGFR2 targeted MB: herpes simplex virus – thymidine kinase/ganciclovir-loaded vascular endothelial growth factor receptor 2 targeted microbubble; IgG: immunoglobulin G; IgM: immunoglobulin M; IL-12: interleukin-12; MB: microbubble; NP: nanoparticle; NC: nanocluster; PET: positron emission tomography; shRNA: short hairpin RNA; SPECT: single photon emission computed tomography; SPIO: superparamagnetic iron oxide; SUVR: standardized uptake value ratio; WBC: white blood cell

expression. Quantitative (e.g. HPLC) as opposed to qualitative (e.g. immunohistochemistry) methodologies, in general, are more superior in terms of precision and accuracy. Results for FUS delivered therapeutics are summarized in Table 1, and annotated in detail with the animal model, FUS and MB parameters, and measurement technique in Table S2. Unless otherwise stated, the results were found in healthy animals.

Despite the abundant evidence that FUS-induced BBB opening can enhance CNS delivery of diverse therapeutics, the meaningful characteristics for the relative efficacy for a given compound are less clear. Nevertheless, evidence from our search, as well as from studies aimed to optimize CNS delivery in general offer insight into the benefits and limitations of FUS. An important determining factor is the size of the therapeutic (Fig. 4). The relative improvement in penetration of fluorescently-labelled dextran of various sizes after FUS is inversely related to their molecular weight [25,85,86]. Similar results have also been obtained from studies involving fluorescently-labelled liposomes ranging in diameter from 55-200nm [87]. These outcomes are consistent with work suggesting that FUS-induced BBB opening produces paracellular gaps up to approximately 65nm, and that time-dependent closure of such openings ensures smaller windows of opportunity for drug delivery as the size of therapeutics increases [88]. This was validated by the temporal extravasation dynamics of magnetic resonance contrast agents with varying hydrodynamic diameters.

Although paracellular routes from disrupted tight junctions are emphasized as the principle mechanism, transcellular routes also appear to contribute to FUS-mediated BBB disruption. Firstly, FUS stimulates caveolae-mediated transcytosis, which is otherwise suppressed in the BBB [89], based on the distribution of tracers within caveolae-like membrane invaginations and intracellular compartments [90,91]. While the typical dimensions of caveolae are in the range of 50-70nm [92], caveolae-mediated transcytosis may contribute to large particle transport in light of previous studies in nanoparticle delivery and transcellular $T_{H1}$ cell diapedesis [92,93]. Secondly, sonoporation of cellular membranes may provide an additional route of transcellular passage. However, *in vivo* evidence is lacking. The pore sizes range from tens of nanometers up to the micrometer scale as assessed by several techniques, including the fitting of patch clamp data to electro-diffusion

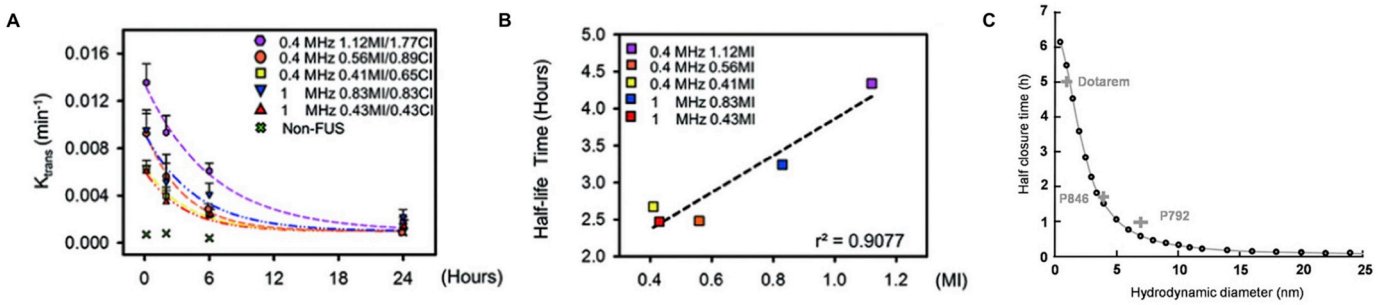


**Fig. 4.** (A) $K_{trans}$, measure of capillary permeability on dynamic contrast-enhanced MRI, over time for various mechanical index MI /cavitation index CI tested to induce BBB opening. MI is defined as peak negative acoustic pressure over the square root of the frequency, and CI as peak negative acoustic pressure over frequency. (B) MI and half-life of $K_{trans}$ after BBB opening appear to be linearly correlated. (C) A theoretical model to predict the duration of BBB passage for various hydrodynamic diameters. Half closure time is defined as the time after BBB opening to achieve 50% delivery of maximal dose at the sonicated region. Experimental data with different contrast agents in this MRI study are represented by grey crosses. Dotarem has a hydrodynamic diameter of 1nm, P846 4nm, P792 7nm. (A) and (B) are reprinted with permission from Chu et al *Scientific Reports* 2016. (C) is reprinted with permission from Marty et al. *Journal of Cerebral Blood Flow & Metabolism* 2012.

models, real-time imaging, and electron microscopy [94]. Lastly, FUS can temporarily reduce P-glycoprotein expression [24,95], suggesting drugs typically effluxed by this transporter might experience higher relative increases in local concentration within the parenchyma.

Other drug characteristics - charge, lipophilicity, BBB interactions, and addition of delivery vector (e.g. nanoparticles) - are also important determining factors for the relative increase in concentration and tissue penetration after FUS-induced BBB opening [96]. Given the aforementioned limitations on FUS for CNS drug delivery, we speculate drug or delivery vector design can leverage these properties, such as by the inclusion of positively-charged motifs to promote adsorption to negatively-charged luminal surface of blood vessels, or by protein and synthetic polymer modifications to increase affinity to components of caveolae [97] [92,98]. For instance, PEGylation effectively further improved penetration of plasmid-loaded nanoparticles through the dense, negatively charged extracellular brain parenchyma after FUS [99]. Future studies should more precisely characterize the effect of FUS on transcellular transport and aim to exploit these routes in drug design strategies.

In addition to the characteristics of deliverables, the local vasculature is a critical determinant of the kinetics and extent of drug delivery following FUS-induced BBB opening. Previous studies classify the kinetics of BBB leakage following FUS into two distinct modes: (i) a fast leakage characterized by rapid dye extravasation from point sources beginning within one minute of sonication and either diminishing within seconds or continuing in a sustained manner thereafter, and (ii) a slow leakage beginning several minutes after sonication and occurring throughout the length of the vessel [34]. Notably, the relative contribution of each of these leakage modes was dependent upon vessel characteristics and associated pathologies. Indeed, a transgenic mouse model of AD exhibited significantly less fast leakage than non-transgenic littermates. Particularly among vessels with associated amyloid plaques, slow leakage dominated with a considerably lower total permeability. In contrast, fast leakage was the predominant mode in non-transgenic mice, especially in the smaller vessels. Additionally, leakage after sonication was consistently associated with a greater increase in vessel diameter in normal vessels than in plaque burdened vessels of transgenic mice. Therefore, one must consider the characteristics of the local vasculature and the effect of any relevant pathology, in addition to properties of the therapeutic, when attempting to optimize sonication parameters for drug delivery.

Although many studies have characterized the safety profile of FUS-induced BBB opening or its ability to enhance therapeutic delivery to the brain parenchyma, fewer studies have addressed the clinically acceptable balance between safety and efficacy in the context of specific diseases. Moreover, distinguishing between more subtle deteriorations from the natural course of the disease (neurodegenerative diseases in particular) or FUS-induced changes can be challenging. In a 6-OHDA rat model of PD, brain-penetrating nanoparticles and FUS successfully delivered GDNF expression vectors to the striatum, resulting in a sustained increase in GDNF expression, substantially reduced dopaminergic cell loss, and improved motor deficits without producing further histological damage [99]. This study also highlights the added benefits of utilizing rationally and specifically designed delivery vectors: fewer doses and necessary FUS-induced BBB disruptions, as well as improved drug penetration once past the BBB. In neuro-oncology, substantial efficacy of enhanced therapeutic delivery with FUS with the near elimination of tumors was demonstrated in many cases [39,59,100–102]. Here, the clinically acceptable level of histological damage is arguably higher. One should note that the safety data presented in these studies are overall weaker, as histological changes typically considered pathological (e.g. apoptosis, necrosis, leukocyte infiltration) might also be indicative of treatment response or tumor progression.

## 6. Limitations and future directions

Here, we conducted a comprehensive review of the preclinical and clinical literature pertaining to primarily the safety and secondarily the efficacy of FUS-induced BBB opening. There are several notable limitations. First, our primary focus was safety. Thus any study without data directly relevant to safety was excluded. This may be the case where the study objective was to demonstrate efficacy. Second, there is substantial heterogeneity in the quality of experimental reporting and results, thereby limiting quantitative analysis of the evidence by statistical summary across studies. Nonetheless, we were able to present the evolution of the field's understanding of how experimental settings might be optimized for safety and enhanced drug delivery. We further provide a database which current and future researchers might utilize as a springboard for future investigations.

Indeed, FUS offers many advantages over existing brain delivery strategies, as discussed previously. Choosing the optimal strategy depends on the characteristics of the platform balanced with those of the deliverable and underlying illness. Promising results from preclinical and pilot human studies have led to ongoing clinical investigations of its safety for AD and ALS without a drug (NCT03321487, NCT03739905), as well as its potential efficacy when combined with chemotherapies for primary brain tumors and metastasis (NCT03616860, NCT003712293, NCT03714243, NCT03626896, NCT03744026). The more rapid progress in neuro-oncology underscores a higher tolerance of the risk-to-benefit ratio than for neurodegenerative disorders. FUS provides flexible and precise spatial targeting, which is a significant advantage in delivering therapeutics to more focal pathologies - such as brain tumors, ALS, PD, Huntington's

disease - while minimizing systemic side-effects. Furthermore, while the safety of repeated FUS-induced BBB opening has been shown in both animal and human studies, FUS is arguably best suited for therapies with sparse dosing regimen (e.g. gene therapies), thereby minimizing risks from chronic exposure [103]. Finally, apart from these considerations, more preclinical studies that directly and quantitatively compare FUS with other brain delivery methods would be highly valuable for choosing between the various BBB technologies [104].

Moving forward, several barriers to successful clinical translation and adoption of the FUS need to be addressed. In addition to technical and engineering innovations for faster and more streamlined procedures, characterizating the long-term safety of chronicrepeated BBB openings and pharmacokinetics of the delivered drug in human subjects should be a priority. Currently, drug penetration after FUS is inferred from surrogate markers such as gadobutrol, which at approximately 550 Da does not accurately reflect the size and chemical properties of relevant therapeutics. More informative measurements can be collected through direct tissue and fluid sampling or nuclear medicine imaging. Finally, long-term strategies to determine the optimal therapeutic and appropriate vehicle (e.g. gene vectors, nanoparticle conjugation) will necessitate a disease-specific approach and effective partnerships with pharmaceutical industries and regulatory agencies.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2019.07.023.

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