Focused Ultrasound
Bench to Bedside

Opportunities to open the Blood Brain Barrier
for therapeutic delivery and liquid biopsy
for Glioblastoma

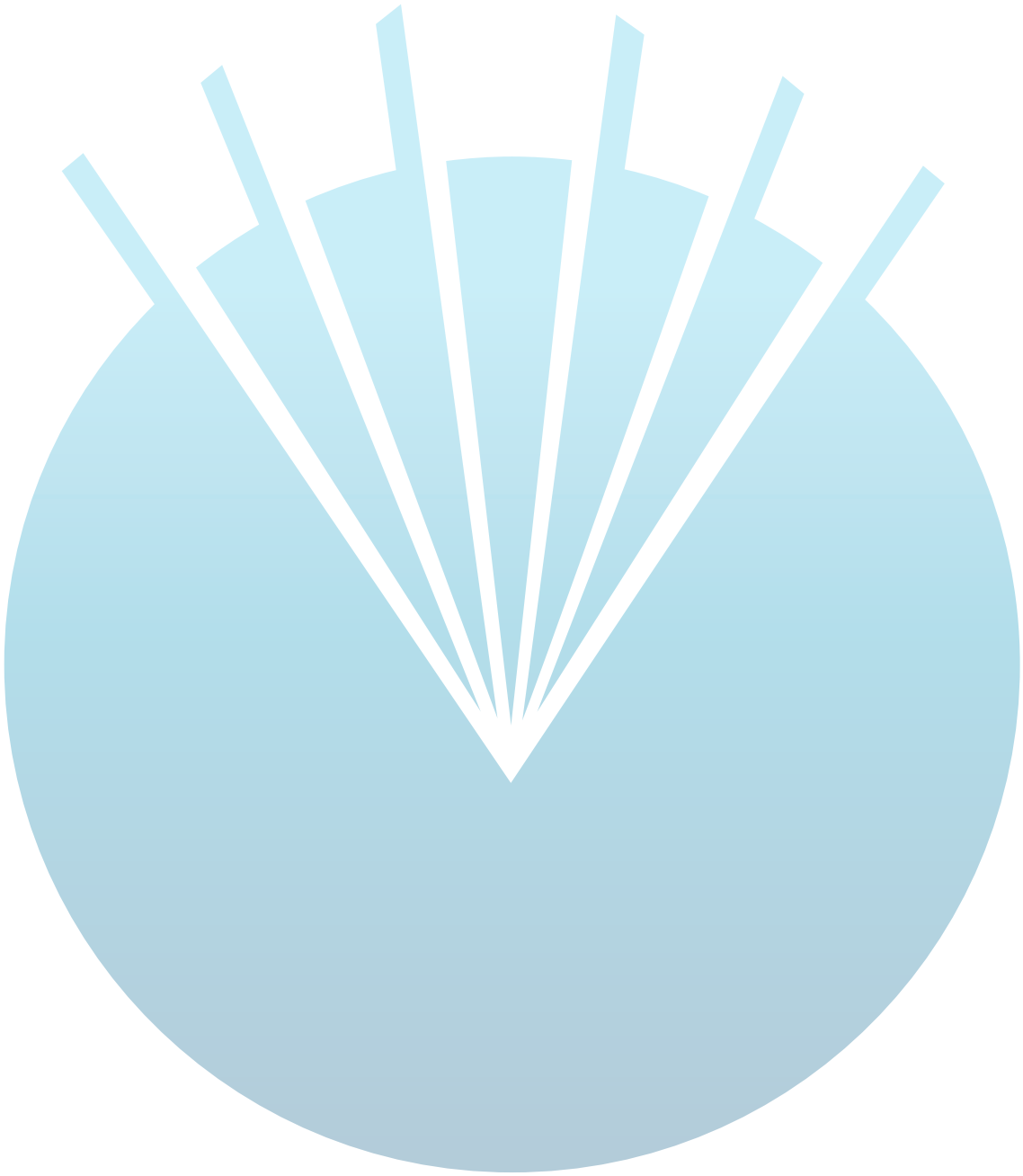
Workshop white paper

6–7 March 2024
UVA Darden Sands Family Grounds
Arlington VA

Sponsored by

Focused Ultrasound Foundation

The Sontag Foundation



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Executive Summary

The Focused Ultrasound Foundation, in partnership with The Sontag Foundation, convened a specialized workshop titled "Opportunities to Open the Blood Brain Barrier (BBB) for Therapeutic Delivery and Liquid Biopsy for Glioblastoma" at the UVA Darden Sands Family Grounds in Arlington, Virginia, on March 6–7, 2024.

This exclusive, invitation-only workshop, organized under the guidance of a distinguished steering committee—including Graeme Woodworth, MD, Susan Chang, MD, Chetan Bettegowda, MD, PhD, and Costas Arvanitis, PhD—was co-chaired by Hilary Keeley, Scott Davis, PhD, Lauren Powlovich, MD, MBA, Suzanne Leblang, MD, and Emily White, MD. It brought together 30 leading experts from diverse fields such as neurosurgery, neuro-oncology, radiation oncology, neuroradiology, industry, and regulatory bodies. The workshop aimed to foster a collaborative atmosphere for knowledge exchange, critically evaluate existing evidence, gauge the significance of ongoing research, and address pressing questions in the field.

The workshop spanned one full day focused on the application of BBB opening for therapeutic drug delivery, and a subsequent half day dedicated to focused ultrasound-enhanced liquid biopsy techniques. Each of the 10 sessions was structured around a "burning question" posed by a steering committee member that facilitated an interactive dialogue among a panel of 4–5 experts, followed by broader participation from all attendees.

Important features of the meeting included transparent information sharing and the cultivation of new collaborative relationships among participants during informal networking breaks. The dynamic discussions underscored a palpable enthusiasm and potential for incorporating focused ultrasound-mediated BBB opening into the therapeutic arsenal against glioblastoma. The participants identified critical knowledge gaps and proposed a strategic roadmap outlining actionable steps to advance our understanding of focused ultrasound opening in drug delivery and liquid biopsy contexts.

Acknowledging the limitations of preclinical models in accurately reflecting the heterogeneity and invasiveness of glioblastomas, the workshop emphasized the importance of conducting additional window-of-opportunity (WoO) trials to gather essential data on the efficacy of focused ultrasound-enhanced therapies. Moreover, it was highlighted that clinical trials should strategically select therapeutics and thoroughly understand their pharmacokinetics when used in tandem with focused ultrasound for enhanced drug delivery. Establishing standardized reporting protocols, encompassing technical parameters, detection of BBB opening, and confirmation of drug delivery, was deemed crucial. Similarly, the advancement of focused ultrasound-enhanced liquid biopsy techniques would benefit from standardized reporting to better understand the relationship between BBB opening and the qualitative and quantitative detection of biomarkers in peripheral circulation or cerebrospinal fluid.

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Welcome and Introduction

Lauren Powlovich and **Suzanne Leblang** welcomed the attendees and thanked the steering committee for their time and expertise in creating the agenda for an engaging workshop. Dr. Leblang reminded the audience that the Focused Ultrasound Foundation previously held a workshop on GBM in 2021. Dr. Powlovich provided an overview of the structure of the workshop.

FUS BBBO Parameters

Burning Question 1

Which FUS BBBO parameters should be reported/aligned to unify language and allow for comparison across devices and enable more effective communication between researchers, industry, and clinicians?

Moderator
Costas Arvanitis

Panelists
Kullervo Hynynen, Elisa Konofagou, Nathan McDannold

A better understanding of the parameters used in preclinical and clinical studies of FUS could help clinicians and researchers compare and interpret FUS research results. Some of the key parameters that are generally addressed when using FUS, along with their typical values, include the following:

- Acoustic pressure: less than 1 MPa; generally, about 0.3–0.4 MPa peak negative.[1] This is at the lower end of the pressures used in diagnostic ultrasound.[1]
- Pulse duration: about 1–10 ms. This is 1000 times more pulses than those used in diagnostic ultrasound.[1]
- Pulse repetition frequency (PRF): about 1–10 Hz.[2]
- Microbubbles: 1–3 μm. The larger the bubble size, the more significant the biological effects for a given pressure.[3]
- Transducer properties: $f \leq 1$ MHz.[4] These frequences are at the very low end of diagnostic ultrasound. Low frequency is needed for higher transmission through the skull.

  Also, note that vascularization varies in different regions of the brain, which affects transmission and absorption.[5]
- Acoustic emissions: The strength of sound generated by the microbubbles can be measured during sonications.[6]
- K^{trans} amplitude from DCE (Dynamic contrast-enhanced) MRI correlates with BBB opening and amount of drug delivery in nonleaky parts of the brain/tumor.[7]
- Recommendations for reporting therapeutic ultrasound treatment parameters have been proposed as useful guidance for clinicians and researchers.[8] When considering a more specific recommendation for FUS blood-brain barrier opening, possible content could include the above-listed parameters along with addressing technical factors such as cavitation monitoring, the number and frequency of the transducers,

the burst pulse length, mechanical index (MI), power and intensity of the device, hydrophone measurements, MB (microbubble) dose, type, and infusion method, and daily quality assurance. Clinical factors to consider reporting include the volume of the BBBO compared to the targeted volume, the type of image guidance used (i.e., MRI, neuronavigation, no image guidance), the location of the transducer, the duration of sonication, and the measurement of induced bioeffects.[8,9] The goal for standardization of reporting parameters is to accelerate technological advancements, to improve treatments by gleaning knowledge, and thus be able to increase total treatment volume and make treatments faster with improved efficacy.

Essential Data to Include in Clinical Trials

- The pre-, intra, and post-procedure MRI images are essential for visualizing the target and assessing the degree of BBB opening.
- Real-time cavitation monitoring and ensuring that the bubbles are properly injected are also important. Comparing two studies using different devices is difficult. There are no good standards for monitoring cavitation activity. If the devices are the same, the exposure parameters could be examined first.
  - The skull and underlying tissue vascularity must be considered when monitoring cavitation and determining the necessary sensitivity of the detector. With gliomas, white matter is the most likely target because glioma cells tend to infiltrate there. Due to the small size of the bubbles produced in white matter, a very sensitive cavitation detector is needed.
  - Every sonication is different, so we need reliable cavitation monitoring and a standard calibration of cavitation detectors, and the effects vary from person to person.
  - Currently, there are too many differences between bubbles and machines to create a standardized cavitation dose.
  - Simulation models can be important in both the preclinical and clinical settings to compare predicted versus actual sonication location and volume. Although not perfect, the models can help determine how much transcranial energy passes through the skull and where it lands.
- The angle and orientation of the transducer are important for each individual skull, and thus, simulations are critical.
- The mechanical index (MI) combines frequency and peak negative pressure and can be emphasized as a suggested parameter to report. Levels of .4 are safe, and .8 enters a higher risk zone for bleeding. MI does not include pulse duration in its calculation, but pulse duration has also been found to facilitate delivery at large molecular weights such as AAV at low MI.[10]
- Modifying the pulse duration allows for staying at a lower MI while continuing to sustain the cavitation. To deliver large molecules we can use higher PNP but risk higher MI and safety concerns.[11]

- Bubble type, distribution, and concentration are also important considerations. Every bubble is different and works differently. Magnitude signal variation is a problem with cavitation monitoring. Some systems use subharmonic emissions that can help standardize by calibrating that every spot has the same pressure amplitude. There are also huge differences in how bubbles are administered (i.e., infusion, drip irrigation). In clinical settings, an infusion is needed to obtain a consistent concentration and uniform distribution while sonicating. Infusion also compensates for the fast kinetics of bubbles. There are clinical and regulatory limitations on how many bubbles can be given and how fast.
  - Bubbles have been designed for diagnostic imaging over the past 30 years. It is time to encourage manufacturers to perfect the bubbles for therapeutics. The regulatory process may be difficult but not impossible. Cavitation cannot be detected well at a low dose. Many sonications are needed due to the current limit on the amount of MB's injected and we use lower doses compared to the preclinical animal studies. Also consider that one may need to sonicate repeatedly over the same beam path. Overall, we may need to use lower doses during a continuous infusion to spread out the dose over a longer time and to target larger volumes.
  - Research is warranted to note if there is a need for uniform bubbles with specific shells.
  - FUSF has a Microbubble working group to move this field forwards and is nurturing relationships with commercial entities to develop therapeutic bubbles.
- A generally accepted quality assurance (QA) phantom is needed. The American Association of Physicists in Medicine (AAPM) has developed a QA phantom for high-intensity focused ultrasound (HIFU).[12] Its use will depend on the research goal. For example, the phantom would not be sufficient for evaluating bubble interactions. However, the QA process could be improved by including microbubbles in the phantom.
  - Real-time robust feedback with MRI scanning is better than a phantom, as every patient's skull, bubble distribution, etc., is different. With MR-guided systems, we know immediately about BBBO with gadolinium enhancement and T2* changes. Using a diagnostic and therapeutic transducer together may also provide real-time feedback.
  - We need better planning systems like Radiation Oncology (please note that DQA for a gamma knife is different than a Cyberknife, and thus, different FUS machines may need different phantoms): As Radiation Oncology QA depicts location and dose, FUS DQA should report target location and dose, taking into consideration heterogeneity of drug distribution after BBBO.
  - Although a unified reporting of input parameters for a variety of devices is needed, focusing too much on standardization could detract from the result, which is confirming the opening of the BBB. Results needto be published in a way that is meaningful to others.

- Other considerations:
  - Parameters change during a clinical trial and are modified even within each patient to adjust for safety and efficacy and prove we are getting the drug across the BBB (proven in preclinical trials), but the translation to humans is complicated, although it has been reproducible across species.
  - Devices are continuously improving but are not optimal yet. Better devices will be key for reducing variability, but some variability is due to the patient's anatomy and the vascularity of the target. Currently, FUS BBBO can target 70–100 cm3 but new software will improve that volume and open even more if we can give more MB.

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BBBO Measurement and Therapy Delivery

Burning Question 2

How do we measure BBBO and therapy delivery beyond Gadolinium enhancement?

Moderator
Graeme Woodworth

Panelists
Nathan McDannold, Ali Nabavizadeh, Kazim Narsinh, Robert Thorne

Standardizing ultrasound to a given protocol or device may seem complicated but it is possible. The terminology (e.g., pulse repetition, frequency) can be simplified quickly to allow for the discussion of "focused ultrasound doses" for a particular device. The energy of ultrasound can be dosed through the skull and measured. However, the bio-effects of that energy are not as well-known and depend on the context and the target tissue.

The safety, feasibility, and repeatability of FUS+MB have been established for brain tumors with multiple devices. When considering FUS for opening the BBB in gliomas, the stage of the disease is key. Treating a patient with a totally resected glioblastoma with residual disease, for example, is very different than treating a patient with a recurrent tumor that has already had radiation, chemotherapy, or surgery. The ultrasound settings and the end bioeffects will differ for each situation. The challenges now are to determine how to identify therapies (either in combination with FUS or with FUS alone), standardize the treatments, and launch clinical trials in a systematic way. The devices currently in clinical use differ from one another and may require separate QA processes.

The moderator asked the panelists to discuss the measurement of the bioeffects of FUS+MB when used for opening the BBB and how FUS+MB may affect both drug delivery and therapeutic efficacy. Possible considerations for measuring the BBB opening include:

1. Acoustic emissions of microbubble activity
2. MRI scans with Dynamic Contrast-Enhanced (DCE) MR Perfusion with DCE K-Trans measuring capillary permeability
3. Radiolabeled Positron Emission Tomography (PET) imaging
4. Serum biomarkers
5. Direct tissue analyses ("window of opportunity" (WoO) studies)
6. Intravital microscopy[1]

Measurement of BBBO

- Imaging with MRI and Gadolinium: As there is a size-dependent permeability change of BBB, using Gd (600 Daltons) is not a good surrogate when therapeutics such as Ab's may be larger at 150 K Dalton. Another way to compare sizes is Gd <1nm, Ab 10nm, and AAV 22nm. Note technical differences in the amount of Gd enhancement also depend on the timing of imaging after Gd injection.
  - There are nuances to using gadolinium enhancement as a surrogate for assessing drug delivery across the BBB. "One opening does not equal another opening." The size of the opening may vary depending on pressure changes or cavitation dose.
- Monitoring with Cavitation: As technology and microbubble doses change and areas of the brain have different inherent structures, we adjust parameter settings during trials and need to prove BBBO for each sonication. Current devices monitor in real-time, so we have a standardized protocol for that device. The cavitation dose prescribed may not be achieved due to lack of resonance of bubbles, etc.
  - Cavitation imaging with a receiver array can detect the location and size of BBBO. Stable cavitation indicators use higher harmonics to tune the opening and measure to levels without bubbles.
  - FUS BBBO opens peri- and transcellular pathways and deactivates P glycoprotein efflux pumps, but we cannot currently preferentially activate any of these pathways. Radiation therapy affects the tumor (medulloblastoma) vasculature differently than the surrounding normal vasculature by upregulating P-selectin, cav1 trafficking, and transcellular pathways. We cannot yet decipher BBBO pathways and the size of BBBO.
- Newer imaging techniques are limited:
  - An optimal imaging technique needs to have high resolution and sensitivity.
  - DCE can be done but needs unified guidelines, which are ongoing.
  - Photoacoustic imaging, which involves light (laser) in and sound out, is possible, but poor resolution and penetration through bone are limitingissues, so it may need a craniectomy defect. Possibly test it with more superficial tumors like the temporal lobe.
  - Magnetic particle imaging can detect nanoparticle tracers with high resolution and sensitivity. These nanoparticles are attached to a therapeutic, so measurement of delivery is specific to the agent.
  - Need to scale and move outside the MRI scanner.
- Potential Serum biomarkers:
  - Are there markers that can predict if there is BBBO, and how large is the FUS BBBO itself? Can we create a fingerprint from the patient before and after FUS BBBO, like a blood sugar test, and obtain quantitative measures from the mechanical perturbation that would inform whether there is BBBO or not? We could also create a color map of the size of the opening that would help direct the type of therapeutic that could be delivered, Ab or virus, etc.

Measurement of Drug Delivery and Subsequent Therapeutic Effects

- Future therapies will likely be antibodies, which generally have long half-lives. Preclinical data showing that the antibody is delivered into a healthy brain may not be enough to determine the dose needed to treat a human brain tumor and extend into the infiltrating margin. Studies should be designed to extract maximal information from radiolabeling, surgical excision, and contrast enhancement.
- Direct radio-labeled drugs are the best current option but have limitations. It is expensive and associated with radiation. As we cannot track in real-time, we are unable to tweak BBBO parameters to optimize delivery. Other limitations include that only 2 drugs can be labeled at the same time, the delay in PET scan imaging after delivery may not reflect the exact measurement as one also needs to consider the ½ life of the drug, there are changes in washout (glymphatic) mechanics, and BBBO closure times are different. The FDA may consider it a drug-device combination, which is a challenge, and an Investigational New Drug (IND) application would be needed for every drug used.
- Window of opportunity (WoO) studies should also be considered when designing a trial with FUS BBBO in combination with a therapeutic agent. Include a spatial analysis of the tissue after surgical removal and compare the specimen to the imaging data obtained during the FUS procedure.
- Questions remain regarding what are the bioeffects of the BBBO itself and the sterile inflammatory response?

Reference

1 Schoen S, Jr., Kilinc MS, Lee H, et al. Towards controlled drug delivery in brain tumors with microbubble-enhanced focused ultrasound. *Adv Drug Deliv Rev*. Jan 2022;180:114043. doi:10.1016/j.addr.2021.114043

Optimizing Therapeutics with FUS BBBO Parameters

Burning Question 3

How can we optimize efficacy and synergy by combining therapeutics with FUS BBBO parameters?

Moderator
Costas Arvanitis

Panelists
Kullervo Hynynen, Elisa Konofagou, Jann Sarkaria, Graeme Woodworth

Many questions remain about how to optimize FUS BBB opening parameters when combining FUS with various types of therapeutics. Researchers need to address the strength of the BBB opening with FUS (less than 100 nm), the duration of therapeutic delivery through the opening (about 4 to 6 hours), and the targeting precision (about 1–2 mm).

Chemotherapy
Due to the high toxicity of chemotherapeutic agents, targeting precision must be good. The BBB opening size is compatible with all chemotherapeutics, and the timing of delivery would be good, considering the drugs' pharmacokinetics.

Antibody
The strength of the opening would be sufficient for most antibodies, and the timing of delivery would be much less (greater than one day). Drug toxicity is considered low.

Nanoparticles
The strength of the opening needed would depend on the selected nanoparticle. The timing of delivery would be comparable to the treatment's pharmacokinetics and there is low drug toxicity.

Viruses
The strength of the opening would depend on the capsid. The timing of delivery would be comparable to the treatment's pharmacokinetics, with an expected moderate drug toxicity.

The moderator asked the panelists to discuss how the FUS parameters interface with various drugs and drug classes. Should potential therapeutics for combination with FUS be grouped into these broad categories when considering the FUS parameters, or should each agent be viewed individually? The panelists were asked to consider whether currently used drugs were sufficient for clinical testing and how trials could be designed to enhance the delivery and impact of the drug in the tumor and its microenvironment. They discussed whether there were missed opportunities to repurpose drugs with unfavorable

Figure 1

Optimization of FUS parameters for BBBO

How do we optimize efficacy (and synergy) by combining therapeutic FUS BBBO parameters? Do we need to optimize the FUS BBBO for each drug?

- Optimize the "strength" of BBBO
- Optimize the therapeutic delivery timing
- Optimize the targeting precision

FUS BBBO	Chemotherapy	Antibody (Ab)	Nanoparticle (NP)	Virus
Strength (<100 nm)	Sufficient for all **Excellent**	Sufficient for most Abs **Good**	It depends on NP **Modest to good**	It depends on Capsid **Modest to good**
Duration (x4–6 hours)	More than good drug PK **Good**	Much less than Ab PK **Modest** (>1 day)	Comparable to drug **Good**	Comparable to drug PK **Good**
Targeting (1–2 mm)	Needs to be good due to high drug toxicity.	Low drug toxicity but what about antibody-chemo conjugates?	Low drug toxicity but what about NP-chemo conjugates?	Moderate drug toxicity (?)

pharmacokinetic and pharmacodynamic properties and whether a combination treatment strategy should be adapted to the targeted region (i.e., tumor core vs. infiltrations) or to the tumor phenotype (i.e., immune priming synergy with drug conjugates).

Protocols

- A clinical trial of a combination therapy with FUS should include
  1) a standardized protocol for the FUS+MB procedure,
  2) preclinical or clinical evidence that the drug gets in (radiolabeling or WoO studies) and
  3) applications in the same general context of the disease (i.e., recurrent vs. resected glioblastoma).
- Maximum drug delivery occurs during the sonication, so FUS+MB should be performed when a maximum concentration of drug is available.
  - It is important to understand the drug's pharmacokinetics and half-life, how well it passes through the BBB, and where it can be used optimally. For example, in the study with DIPG, as the drug Panobinostat was administered every other day, FUS BBBO was performed every other day with the drug to maximize delivery.

  - The actual duration of FUS BBBO still needs to be determined for which target area, as this may change based on the properties of the tumor and location.
  - It is possible that a drug's Cmax is driving efficacy, but drug exposure over time may matter more with agents with a short half-life and may require a longer duration of the opening.

- Is there enough preclinical evidence that we can translate specific molecules that have facilitated trans vascular transport? Radiation studies show changes in E-selectin and ICAM 1, endothelial markers of inflammation, that can help with targeting therapeutic agents. Similar effects can be shown with FUS to help localize drugs to the endothelium at the site of FUS. We can research this with antibody-drug conjugates (ADCs). Does it bind? Does it transcytose? Does it go through the interstitium?
- Questions also remain about treating the tumor core versus the tumor periphery. With radiation therapy, the densest portion of the tumors gets the highest dose, targeting the core. The surgical approach is to resect the core and target the periphery. However, a major concern is assuming that the residual tissue has a similar biology to the resected tumor specimen and making chemotherapy choices based on that flawed assumption. This issue is relevant to drug selection for and delivery to invasive GBM cells.
- Other considerations: The location and density of the blood vessels may determine how to optimize drug distribution and retention further away from these blood vessels. Crossing the BBB is not the only challenge.
- A multi-modal approach to treating glioblastoma is needed and FUS is one strategy that can help but we will need to treat the whole brain. There are significant advantages to using FUS for the entire brain rather than something like mannitol as we can more uniformly open the BBBO which is heterogeneous to begin with, and even more heterogeneous due to tumor. We can also control the opening, and tailor the dose and resulting bioeffects to specific areas of the brain.

Choice of therapeutic

- Consider drugs that do not penetrate the BBB without FUS that show promise in the lab. Drugs such as antibody therapies that have reasonable safety profiles for other tumors but have not been studied in brain cancer may be the first to be explored. Nanomedicines may be next, followed by cellular therapeutics such as CAR-T.
- The first steps may be to identify an "amazing drug" in preclinical studies, conduct safety studies, and then see whether its delivery can be improved with FUS.
- FUS may play a role in the delivery of gene therapy (with nanoparticles or viral vectors).
- Consider using other delivery modes with FUS, such as intraarterial or intranasal, to decrease systemic toxicity and still enhance delivery.

- Need a drug with good PK/PD that stays in the tumor with limited efflux while considering how often FUS BBBO will be performed to deliver the drug to the target.
- Also, consider limiting toxicity as microtubular agents have neuropathy complications.

Immune considerations of FUS BBBO and "priming" of the tumor microenvironment

- What does FUS+MB do to the brain other than open the BBB? Be careful with the terminology as BBB disruption is associated with pathology such as Alzheimer's disease, so use the term BBB opening. There is an immune response with gene expression in microglia, oligodendrocytes, and macrophages, which peaks in 72 hours and is alerted to existing pathology. Studies show that immune cells do not digest the drug, but this area needs more research as not all cells respond the same way the same of different drugs.
- FUS alone and FUS+MB without BBB opening does alert the immune cells, but they are less compared to when the BBB is opened with FUS + MB, so they are likely secondary to mechanical events. There is a linear correlation of FUS strength with cavitation and immune effects.
- Recruitment of MDSCs (myeloid-derived suppressor cells) or T suppressor cells needs to be studied by applying FUS in a specific way that is repeatable.
- FUS BBBO can allow plasma proteins to stimulate immune cells to enter the brain, changing the brain tumor's environment from cold to hot.

Limitations of preclinical models for immune response and drug delivery

- FUS BBBO alone is equal to no drug in GBM tumor growth preclinically.[1] An in house meta-analysis (unpublished data provided by Dr. Natasha Sheybani) revealed 2 conclusions: studies that have assessed drug delivery in glioblastoma use xenograft models with responses that are not fully understood, and questions remain about how well syngeneic preclinical models (GL261) fully capture the nuanced landscape of the tumor and immune microenvironments in glioblastoma. Many studies also ignore the heterogeneity of the tumor and its environment.
- Very difficult to perform preclinical studies on infiltrative margins.

Reference

1 Schoen S, Jr., Kilinc MS, Lee H, et al. Towards controlled drug delivery in brain tumors with microbubble-enhanced focused ultrasound. *Adv Drug Deliv Rev*. Jan 2022;180:114043. doi:10.1016/j.addr.2021.114043

New Therapeutics to Test for Combination with FUS BBBO

Burning Question 4

What new therapeutics should be tested for combination with FUS BBBO? Which type would you predict would be most successful and why? Which will have high potential in future to impact outcomes?

Moderator
Susan Chang

Panelists
Clark Chen, Nino Chiocca, Jann Sarkaria

A range of therapeutic agents are currently under investigation for combination with FUS.[1] These include:

- Chemotherapy agents,[2] carboplatin and paclitaxel
- Antibodies and antibody-drug conjugates[3]
- Nanoparticles and their various packagings[4]
- Viruses[5]
- Microbubble drug conjugates[6]
- Drug conjugates that use nanoparticles[7]

The goals of treatment are to not only increase the therapeutic index of the drug and deliver more drug to an effective target, but also to spare systemic toxicity. In addition to identifying potential new therapeutics, questions remain about predicting which therapeutic agents will be most successful when combined with FUS. Why are they successful? Which ones will have a high potential to impact future outcomes for patients?

The panelists discussed the type of preclinical data needed to support clinical trial evaluation, methods for assessing when and how long to administer an agent, selecting the tumor volume (contrast enhanced [CE] and non-contrast enhanced [NCE]) components, and assessing coverage, delivery, and drug distribution.

Choose Therapy Where Enhanced Delivery is Needed

- Researchers need to identify an amazing therapy where delivery is a fundamental issue. An MDM-2 inhibitor (Murine Double Minute Clone 2) would be ideal. However, there are currently no FDA-approved MDM-2 inhibitors. There are also concerns about how long the MDM-2 inhibitor would stay once the BBB opening is sealed. Efflux pressure would push the drug out.

- Antibody-drug conjugates (ADCs) such as Depatux-M (Depatuxizumab Mafodotin) have worked well in EGFR (Epidermal Growth Factor Receptor) flank tumors, with heterogeneous results in orthotopic tumors. Efficacy is related to disruption of the BBB and specific patient-derived xenografts (PDXs). Questions remain about the safety of ADCs in normal brain tissue.
- A general approach to designing a clinical trial would be to learn from past mistakes and identify drugs that have compelling data to support combinations. Revisit drugs that worked in the lab but had poor penetration issues due to the BBB. This would convince sponsoring companies that the trial is worth their investment and add on enhanced delivery with FUS.
  - Consider that drug companies do not want to repurpose drugs that failed phase 3 trials and do not want to use drugs with short patent life.
  - Until there is a personalized medicine approach, no single drug will be a "magic bullet."
  - If considering combinatorial therapies—using FUS as just a delivery system may be able to give combinatorial therapy with lower doses to minimize toxicity.
  - Evolving knowledge suggests that FUS is not just a delivery system and exerts a combinatorial therapy: mechanical perturbation of the microenvironment at the target has potential for therapeutic activity beyond just enhancing the drug concentration, such as enhancing immune effects.

Limitations of Preclinical Models

- Moving to clinical trials typically depends on preclinical models, such as mouse models, PDXs (patient-derived xenograft models), and organoids. However, each model has limitations. PDXs, for example, do not have an intact immune system. Each model provides different information, suggesting that several are needed, which is costly and impractical. It may be more valuable to do a Phase 0 window of opportunity study in humans, with tissue from three or four patients, than a mouse study.
- Canine glioma models have been studied, but the tumors are more like oligodendrogliomas than human GBMs. However, some of the targeting and pharmacology are similar.
- Researchers need to ask the right questions of the right models. For example, is the model's immune system or brain size comparable to humans? Is equivalence assumed based on size? How comparable are the BBBs? Are genes evolutionarily conserved?
- Researchers in FUS may be too optimistic about interpreting mouse models, which often provide significant survival benefits. A minimum version of success in a PDX model may be doubling survival, but ideally, it should be tripling or quadrupling. The standards should be much higher.

- FUS devices are different in animals than in humans, which also limits the interpretation of preclinical studies especially when it relates to the combinatorial.
- Preclinical models can still help screen for the best drug and FUS mechanisms of action.

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FUS BBBO-related Toxicities

Burning Question 5

What FUS BBBO-related toxicities should be defined, monitored, and reported?

Moderator
Graeme Woodworth

Panelists
Ali Nabavizadeh, Kazim Narsinh, Patrick Wen

Questions remain about the potential toxicities of agents combined with FUS, FUS +MB, and FUS alone. How should toxicities be measured and monitored? Which effects should be reported? Along with monitoring potential clinical symptoms, such as headache and seizure, other techniques, such as monitoring acoustic emissions of inertial cavitation of microbubbles, may be useful. Questions remain about how to grade the degree of any toxic effect and correlate it with an outcome. Can the effects be categorized as grade 1 or 2, for example, to facilitate communication about the level of an effect? Are there parameters for monitoring bioeffects and ensuring that a treatment is not creating undesired effects in the brain?

Radiographically Detected Potential Toxicities

T2* susceptibility weighted imaging (T2*/SWI) is a technique that has been used to assess the extravasation of hemoglobin products into the tissue at the location. FUS target T2*/SWI can be obtained after treatment or concurrently during the treatment using the Insightec device, which can gather live T2* imaging. Results of a recent study using the Insightec device showed T2* activity in the brain from patients with Grade 2 and 3 gliomas treated with FUS before surgery.[1] Two different types of T2* patterns were noted. In one, immediate development of T2* occurred, which is rare. In the other scenario, which is more typical, there was a slow development of T2* in the area, often absent at the end of the 90-120 second sonication but appeared after several minutes. Upon stereotactic biopsy of these areas, there was no hemorrhage on pathology, and thus, the exact etiology is unclear.

- We need to standardize T2* image acquisition to compare results and tease out the type of blood components (such as met or oxyhemoglobin) that may be released from small capillaries.
- Why do some T2* changes occur early and others late? Does it correlate with the strength of FUS BBBO, inertial cavitation feedback, or underlying tissue differences?

- Is it optimum to tune the system to achieve FUS BBBO without T2* changes? Do T2* changes indicate or confirm FUS BBBO with an improved biological effect?
- If you want to create more BBBO opening, you may repeat the sonication, but do not change pressures.
- Clinicians are not too worried about these T2* changes, as they pale in comparison to damage from surgery and are not associated with any neuronal loss.
- 2D GRE or 3D SWI images can be obtained, and we should develop a scale like the Alzheimer's Disease grading scale for T2* spots in screening and follow-up scans. Compare it to convection-enhanced delivery (CED) grading on follow-up MRI changes.
- 7/33 cases with pre- and post-FUS BBBO scans developed new T2* spots, which are infrequent and are only in very small areas.
- No hemorrhages or immediate clinical consequences have been documented in any patients to date.
- Analyzing FUS parameter data with MRI scans can help build predictive models. Thus, cavitation-based monitoring will be more reliable.
- Radiomics (tumor recurrence prediction maps) and amino acid PET are new technologies being used more in Europe than the U.S. and may be options to consider for targeting and assessing toxicity. Amino acid PET appears to be more robust than radiomics in some studies. 2 PET tracers are going through FDA approval. T2 hyperintensity FLAIR and amino acid PET areas do not necessarily correlate, and amino acid PET better identifies areas of aggressive tumor within the T2 FLAIR areas.

Clinical Evaluation for Potential Long-Term Toxicities

- Questions about long-term effects after repeated FUS BBB opening will be increasingly relevant. Are there long-term cognitive effects, especially when we deliver higher concentrations of therapeutics in specific areas?
- BBB dysfunction is already associated with neurodegenerative diseases.
- CarThera has performed up to 15 sonications in each patient, but it is difficult to know if any issues are related to the disease, side effects of other treatments, or the FUS. Longitudinal neurocognitive testing should be considered.
- Dr. Konofagou shared that her laboratory has unpublished data on cognitive outcomes and MRI scans in non-human primates for over 12 years, with tens to hundreds of FUS BBBO procedures in each primate. She was encouraged to publish this data, which proves safety. Some of the data was submitted to the FDA towards the IDE approval and could be retrieved to include it in a future publication.

- Track patient-reported outcomes. The perspective of the patient must be considered. Research should track what the treatment means for the patient, whether it is easy for them and their caregivers, and whether it is worth it for them. A therapy that is cumbersome to patients and their families will not be used. Some [non-FUS] devices have not been successfully implemented because they are hard on the patient (such as tumor treating fields TTF).

Serum biomarkers

- Serum biomarkers, such as glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), may offer additional options for monitoring toxicities.
- Serum biomarkers of toxicity need further exploration for FUS but have already been researched with other diseases, such as sepsis and traumatic brain injury.

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Clinical Settings for Testing FUS

Burning Question 6

Which clinical settings would be most appropriate to test safety and/or effectiveness of FUS? In which situation would FUS provide more data regarding therapeutic effects?

Moderator
Susan Chang

Panelists
Manmeet Ahluwalia, Chetan Bettegowda, Nino Chiocca, Michael Vogelbaum

Once a potential therapeutic agent is identified to treat glioblastoma (GBM), the challenges for exploring the clinical role of FUS are to

1) determine the most appropriate clinical settings to test effectiveness and safety and
2) identify situations that would provide the best data.

Window of opportunity studies, which take advantage of the window of time between a cancer diagnosis and the initiation of cancer therapy, offer possibilities for gathering data on the use of FUS. In the trajectory of a patient's care, the first step is typically a diagnostic MRI scan, which can offer an opportunity for further exploration with FUS, although this time point before surgery would be difficult as the diagnosis of GBM is not confirmed with pathology. Other time points to consider treating with FUS include post-resection during radiation and/or chemotherapy, adjuvant, post-adjuvant setting, or at/after a recurrence or progression.

The panelists discussed opportunities for when and how to test FUS in clinical settings. Situations that involve a high-grade glioma are difficult for patients and their families. Introducing a clinical trial at initial diagnosis can be challenging especially as patients are symptomatic and may need emergent surgery before starting a WoO trial. Involving them in a clinical trial can add to the emotional stress and can be logistically difficult. Sometimes the idea of a new technology, like FUS or delivering CAR T cells, can also sound very appealing to some patients. It is important to consider the stage of treatment when comparing survival curves for trials. They reviewed possible indications for FUS and suggested potential patient populations to include in clinical trials. Key points included the following:

Window of Opportunity Trials

- Take advantage of the ability to obtain resected tumor tissue from patients undergoing surgery. Such studies may be limited in that they involve just one-time point, but they can address some simple scientific questions and help to understand a drug's delivery and action.

- WoO studies differ from Phase 0 clinical trials.
- A Phase 0 clinical trial is a specific concept created to distinguish proof-of-concept clinical research from therapeutically oriented trials that require an enhanced regulatory process. Phase 0 trials feature a treatment regimen that can impact eligibility for future trials. The Phase 0 concept typically involves a single dose of exposure and an assay to assess pharmacokinetics and pharmacodynamics. Phase 0 trials in neuro-oncology are difficult because they require biopsies and surgery.
- Window-of-opportunity studies use a "Phase 0-like" approach to studying a potential therapeutic dose and its actions. They are usually carried out in settings that can continue the therapy to achieve a potentially beneficial effect. The outcomes data can be paired with the biological data. Conducting such a study in the recurrent setting is good for addressing mechanistic questions and is less difficult than in a newly diagnosed setting.
- SNO encourages more biopsies to support these trials
  - Obtaining serial biopsies could provide more information on how FUS acts as an immunomodulatory tool and that FUS may ignite an immune response in the residual diseased tissue (thalamic GBM's) and follow longitudinally
- Why do some T2* changes occur early and others late? Does it correlate with the strength of FUS BBBO, inertial cavitation feedback, or underlying tissue differences?

Post-surgery Pre-radiation

- 3-week window when patients need to recover from surgery before doing radiation or chemotherapy.

Adjuvant Phase (Post radiation)

- Are the FUS parameters the same in post radiated tissue? Consider that this stage is most promising for the patients and for company investment perspectives.

Recurrent Setting

- This may be the best setting, but no therapy has proven effective at this stage. If it fails here, they won't approve it for the upfront setting either.
- PK/PD of a drug for recurrence can be studied at secondary resection and evaluate target engagement, and effects in the infiltrating and enhancing components.
- When considering eligibility criteria for a study, the patient population needs to be clearly defined to determine whether the intervention is having an effect. The criteria depend on the questions being asked, the current evaluation of the drug, and the drug combination technology. Tumor factors (size, location, etc.) need to be considered.

- When considering eligibility criteria for a study, the patient population needs to be clearly defined to determine whether the intervention is having an effect. The criteria depend on the questions being asked, the current evaluation of the drug, and the drug combination technology. Tumor factors (size, location, etc.) need to be considered.

Other Considerations

- Consider gliomas other than GBM.
- Basket trials, which test drugs in multiple tumor types, could be carried out for brain tumors that have a defined mutation and effective therapies such as ependymomas. However, many of these agents need to be delivered daily so consider agents such as ADC's or monoclonal Ab's that need to be administered every few weeks so logistically it is easier to pair with FUS treatment.
- There are FUS systems that are outside the MRI scanner and thus can be used more frequently. This has been used with pediatric DIPG patients and Etoposide, as patients receive the combined treatment every two days. The patients were treated outside the MRI scanner with no sedation and could be treated in a wheelchair with family present.

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FUS-Enhanced Liquid Biopsy

Introduction

Liquid biopsy has been used in oncology for decades to help understand neoplastic processes without tumor tissue samples. Data obtained from blood and other fluids (e.g., urine, CSF) can help inform different aspects of a cancerous process. For neuro-oncology, the challenge lies in accessing a space that is protected by the blood-brain barrier. The gold standard of diagnosis in neuro-oncology remains surgical sampling, either with minimally invasive procedures such as stereotactic biopsies or with open resection. These approaches are invasive and are associated with risks of anesthesia and neurological injury.

The need for non-invasive methods to understand what is happening in the brain in real-time is paramount—more so than for any other organ system in the body. Since the number of analytes in liquid biopsies is even fewer from central nervous system pathologies due to the blood-brain barrier, there is a need to enhance and enrich the number of analytes in the peripheral circulation or even the cerebrospinal fluid. Focused ultrasound is a technology platform with a variety of mechanisms of action and bioeffects and can temporarily open the blood-brain barrier (BBB). Although most focused ultrasound studies with BBB opening are evaluating the safety and efficacy of enhanced drug delivery in a spatially targeted location, there is also the potential to s allow analytes from the tumor and peritumoral region to flow back into the bloodstream for analysis. In 2023, The *British Journal of Cancer* published an article citing opportunities for liquid biopsies in brain research:

> "At the technological front, increasing availability of liquid biopsy analytes in biofluids of brain tumor patients, development of standard and reproducible processes of sample collection, improved specificity and the sensitivity of tumor-associated signal detection, and the employment of analyte-specific tailored downstream analytical and bioinformatics techniques need absolute attention."[1]

In oncology, the standard biomarkers have been protein-based, such as the prostate-specific antigen (PSA) used in screening for prostate cancer. Neuro-oncology has explored glial fibrillary acidic protein (GFAP), which currently does not appear to be sensitive or specific enough for clinical use. The field is now beginning to move towards other potential biomarkers beyond proteomics. The bulk of research focuses on DNA-based material, which allows for exploring different analytes, mutations, copy number changes, fragmentomics, methylation, and other factors. Entire circulating tumor cells can also be detected in fluids. FUS presents the potential for enhancing the release of materials in many organs, including the brain.

In a proof-of-concept study, after implanting GFP-transfected glioma cells in the brain, higher levels of green fluorescent protein (GFP) were detected in the blood of mice after disrupting the BBB with focused ultrasound and microbubbles (MB).[2, 3] Similar enhanced

levels of GBM-specific biomarkers such as EGFRvIII and TERT mutations were also detected in a mouse model post-blood-brain barrier opening with FUS+MB. In larger animal models, a study in healthy pigs confirmed these findings with increased release of brain-specific biomarkers (GFAP and myelin basic protein [MBP]) in blood collected after FUS.[4] Finally, in a pig glioblastoma multiforme (GBM) model, FUS-enabled liquid biopsy improved the detection sensitivity of two GBM-specific biomarkers (EGFRvIII and telomerase reverse transcriptase [TERT] promoter mutation C228T).[5]

Based on this data, a prospective clinical trial of Sonobiopsy was carried out in five patients with newly diagnosed glioblastoma already scheduled for surgical removal using neuronavigational-guided FUS.[6] Before surgical resection, blood was collected pre- and post-FUS, and then the surgeon obtained tissue samples in the location at both Sonobiopsy targeted and non-targeted brain regions. The focused ultrasound device used for this study was designed so that it directly couples to the navigation probe. Enrichment in circulating tumor DNA (ctDNA) was observed in three of the five patients, and the safety of the procedure was confirmed in a histological analysis of the surgically resected tumors.

Comparable outcomes were also reported in another FUS enhanced liquid biopsy study which demonstrated a 2.6-fold rise in cfDNA, a 3.2-fold increase in extracellular vesicles (ECV), and a 1.4-fold increase in brain specific protein (BSP) in GBM samples following the application of focused ultrasound-enhanced liquid biopsy using an MRgFUS system by Insightec.[7] Others have reported increased release of analytes post FUS in other brain diseases such as Alzheimer's Disease and reported a linear correlation between cavitation dose and the biomarker release.[8]

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Biomarkers for FUS BBBO

Burning Question 7

What biomarkers should be collected to determine FUS BBBO to understand the biology of what is happening during BBBO?

Moderator
Chetan Bettegowda

Panelists
Hong Chen, Nino Chiocca, Natasha Sheybani, Robert Thorne

The panelists discussed various types of biomarkers and their potential for helping to determine whether FUS treatments may have biological and clinical impacts. Key points included the following:

Use Biomarkers to Evaluate the Efficacy and Safety of FUS BBBO

- All potential biomarkers should be considered because it is not known which ones can be released from the brain to the blood. A study design should enable the ability to ask multiple questions on any samples obtained from the study.
  To monitor the safety of FUS BBBO, consider neurofilament (NfL) light chain, which has a very long intracellular half-life of a couple of months, and correlate changes in NfL with the volume of BBBO. Full-length NfL is about 68KDa but one can also measure NfL fragments. Perhaps consider what happens to analytes such as NfL and proteins with FUS thermal ablation and lesioning for movement disorders. A proof-of-concept pilot study showed enrichment of NfL protein concentration in a mouse model with FUS BBBO, but safety and correlation with bleeding or other side effects have not yet been studied. With glioblastoma, however, treatment options are very limited, so some risks may be needed.

Use Biomarkers to Explore FUS BBBO Bioeffects with or without Therapeutic

- Consider narrowing the focus to understand what the FUS beam does to tumor and immune cells near the BBB.
- Select biomarkers that have already been implicated as important, such as those analytes related to immunotherapies/immunomodulation (cytokines, immune cells, etc).
- Some biomarkers (e.g., EGFRvIII and TERT promoter mutations) are very specific for glioblastoma. Questions remain about how well these biomarkers correlate with a particular treatment and how to best assess the correlations.

- The choice of analytes may also be dependent on the stage of disease and goals of evaluating response to therapy, early detection, progression vs pseudoprogression, etc.
- Consider at what time points in a patient's disease course can information change?
- A study of exosomal programmed death-ligand 1 (PD-L1) showed a correlation of patient's blood levels with MRI scans, up to a certain point where likely the increasing amount of necrosis played a role in stabilizing the number of analytes. The Northwestern group described early data that suggested some correlation between progression and increased exosome release.

Standardize FUS BBBO Protocol for the Evaluation of Analytes

- Determining preanalytical variables is a huge unresolved topic that a working group could address. Questions remain about how to process the samples to preserve the sample's integrity for retrospective analysis. BloodPAC guidelines should be incorporated into FUS studies.
- Another pre-analytical variable involves the volume of FUS BBBO. More information and modeling are needed regarding how much volume should be sonicated to increase the amounts of analytes within a specific volume of blood. In addition, more data is needed regarding when the blood should be sampled after sonicating with FUS and different analytes may be released on different timelines. When can differences be reasonably detected?
- The Blood Profiling Atlas in Cancer (BLOODPAC) is a consortium managed by the Center for Computational Science Research, Inc. to accelerate the development and validation of liquid biopsy assays to improve the outcomes of patients with cancer. BLOODPAC developed a Data Commons to enable the sharing of information. Standardization of the data is essential. A working group pared down 55 to only 11 essential technical data elements, which was published in concert with feedback from the FDA. In addition, a set of generic validation protocols for researchers to use was also published to help expedite future test developers to drive ct DNA assays for clinical use. Consider standardizing how to measure the volume of FUS BBBO and use that to normalize results so the field can aggregate data. Key elements to address in establishing a shared protocol may include the typical parameters such as pulse and frequency, as well as equipment standards. The assay must be safe, effective, and reproducible (Assays vary in LOD: .01% LOD–.001%). Need to codify and standardize timepoints of blood sampling.

Other considerations

- Consider using Ommaya reservoirs to obtain serial CSF samples – reasonable to put in the Ommaya in this patient population with no real treatment options.

- We do not need to study CTC's (circulating tumor cells) after FUS as a potential source of metastatic disease as CTCs are released after radiation therapy, surgery (disrupting BBB), and other treatments with no clinical sequelae.
- The CELLSEARCH® CTC test is the only FDA-cleared blood test for circulating tumor cells.
- The future includes obtaining a diagnostic LB while the patient is already receiving a diagnostic MRI—no head shaving is required. The IV for gadolinium is already in place, so just infuse the microbubbles and use MRI pictures to target the area. This likely adds one hour or less to the scan. It is best for the patient if you align FUS LB with treatment cycles and MRI scans.

Analytes to Obtain

Burning Question 8

What analytes should be obtained that may help us understand tumor biology and possibly determine response, recurrence, or residual active disease? Should these analytes be tailored to the specific drug mechanisms of action to measure downstream effects?

Moderator

Chetan Bettegowda

Panelists

Leonara Balaj, Hong Chen, Jim Godsey, Jason Huse, Patrick Wen

Liquid biopsy is a potentially valuable diagnostic and prognostic platform with a promising non-invasive approach for tracking changes in tumor biology. Potential analytes under consideration for investigation in biofluids include: circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), cell-free DNA (cfDNA), circulating cell-free RNA (cfRNA), extracellular vesicles (EVs), total small extracellular vesicle (sEV), tumor-associated platelet's (TEP), and various factors related to genomics, epigenomics, methylation, proteomics (e.g., irinotecan and bevacizumab changes to vascular endothelial growth factor [VEGF] levels), metabolomics, transcriptomics, and fragmentomics.

The moderator asked the panelists to focus on the specific types of analytes that could be obtained with liquid biopsy to help improve our understanding of tumor biology and potentially benefit patients. How can these analytes be tied to therapies and the mechanisms of action of the drugs under consideration for FUS-based therapies? What are some of the leading analytes to consider?

Key discussion points included the following:

- The analytes to study depend on the clinical application and stage of the disease.
- Acquiring baseline samples early in a patient's clinical course is important to understand changes within a patient. 2 clinical scenarios demonstrate the need for noninvasive LB:
  - Patients who want to be in a clinical trial but do not want to risk an invasive tissue biopsy for a definitive diagnosis.
  - Differentiating tumor recurrence vs pseudoprogression. Analytes to consider include methylation profiling and detection of ctDNA and mutation detection. Answering questions about therapeutic changes and finding resistance mechanisms are more complicated. It would be helpful to find resistance mutations like in lung cancer, but temporal heterogeneity is an issue as EGFRvIII may disappear if the drug is not working.

- The most specific marker of glioblastoma is probably chromosome 7 gain 10 loss. EGFRvIII is a good marker but is only seen in about 20% to 30% of tumors. TERT positive mutations are in 60-70% of WT GBM. Finding fibroblast growth factor receptor (FGFR) fusions could open an entirely different branch of diagnostics.
- Liquid biopsy can predict recurrence. Consider using the GeLB score or ddPCR longitudinally. Following IDH-1 quantitative analysis, levels correlate with outcome, as levels that stay high or increase are seen with recurrent disease and levels that linger or drop are noted in responders. At recurrence, TERT, EGFRvIII, and IDH-1 levels were increasing, likely due to more inherent disruption of the BBB.
- It can be challenging to study the entire tumor heterogeneity with transcriptomic/epigenetic changes. Using layered assays and multianalytes will benefit the field. Consider focusing on one constant in the tumor, such as gain 7 loss 10. Focus on commonalities.
- One marker or one mutation may not be the driver of the tumor; the number of copies may be needed, and the biological meaning of the marker are not known. CSF sampling may work well in operable situations and for assessing pseudoprogression and does not require additional technology and costs.

Pros and Cons of Methylation

- Methylation appears to be an accepted method of classifying subtypes of brain cancer from tissue and seems translatable to blood. Methylation [detection] has progressed from bisulfite conversion, which destroys 90% of the target DNA. A process that would enable more target recovery would be valuable. Combining methylation and somatic variant detection in a single assay would be helpful, as well as considering other possible omics, such as fragmentomics and proteomics. Blood-based assays for IDH-1: Current study comparing EGFRvIII in CSF and blood with ddPCR longitudinally-only a 2 mL blood sample is required and is easier. With the other omics, validation studies, and reproducibility with many patients over time are needed. In general, ddPCR can more easily accomplish the same goal with the analysis of 6 mutations and get answers in 2 hours vs methylation which take much longer.
- Methylation test results need to be available quickly to be clinically useful. Methylation is an amazing technology for classifying tumors, but only a few centers in the country have the technology necessary to perform such analyses, and it often takes a month or more to return results. Whether methylation should be considered a gold standard is not entirely clear. Other genomic surrogates could be used for classifying certain tumor entities, and digital PCR. Also, methylation profiling uses the bead array-based analytical system, which is a somewhat outdated technology. Methylation profile is not needed for most brain tumors but can be helpful in specific situations of diagnostic uncertainty, especially in pediatric

tumors. Pragmatic issues prevent the widespread adoption of methylation profiling. Most centers are now using Next Generation Sequencing and can process DNA methylation data but based on the output of older bead arrays and every updated assay, for example, on Illumina, would theoretically change the results of the methylation profiling. Newer platforms run somatic and methylation pipeline. There is a need for fundamental resources to be available to patients in a clinically meaningful time frame.

- Whole genome methylation takes days for results from plasma or CSF (comparing apples to oranges compared to tissue sampling). Additionally, data sets are different, and it would take herculean efforts—would need collaboration for methylation of body fluids: still in discovery phase.
- Recommend a publication using serum and not plasma with glioma epigenetic score (Gelb score quantitative) for classification and possibly for use to differentiate progression from pseudoprogression. Needs standardization and clinical validation.

FUS Considerations

- How much can FUS increase sensitivity and specificity? It is currently in the discovery phase. The industry may be apt to engage in clinical trials if there is a validated LB study to diagnose and follow patients.
- An added value of FUS is the ability to target a specific brain region. Integrating an MR diagnosis with a molecular diagnosis in one platform, without adding to the length of the procedure, will help provide a more precise diagnosis to the patient. The MRI can be used to target specific regions of interest, and due to washout, multiple areas can be queried in a session.
- An easy study would be to compare blood samples with ddPCR assay with and without FUS.
- FUS BBBO may be opening the floodgates but may also stimulate tumors to secrete more biomarkers—need better understanding of MOA—tumor biology.
- The radiographic appearance of tumors does not necessarily correlate with the number of analytes detected. Tumor-suppressive macrophages could decrease the shedding of ctDNA. Post-treatment necrosis can also release more biomarkers. More than structural MRI imaging is needed to correlate with tumor physiology and analytes.
- Well-curated clinical trials are needed. Imaging studies are the current standard of care, but longitudinal liquid biology could also become standard, either with or without FUS. Each measurement provides different information. Being able to do LB even after 1 dose of medication could guide the continuation of treatment or switch treatments—less time would be wasted by avoiding the continuation of treatments that are not effective.
- With stored samples, consider discovery phase interrogation vs tailored studies to correlate with a therapeutic.

The Tumor Barcode

Burning Question 9

How should we study the tumor barcode? High level or specific mutations?

Moderator
Graeme Woodworth

Panelists
Leonara Balaj, Chetan Bettegowda, Jim Godsey, Jason Huse, Natasha Sheybani

FUS-enabled liquid biopsy techniques are being employed to evaluate the release of cell-free DNA (cfDNA) from the brain into the circulation. Analysis of cfDNA can provide information about DNA methylation patterns and fragmentation profiles and can provide insights into the origin and health of the tissue.[1]

Isocitrate dehydrogenase (IDH) status, O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status, and 1p/19q co-deletion are major biomarkers for classifying gliomas. MGMT promoter methylation status is an important prognostic and predictive marker in glioblastoma. However, researchers have not yet established its value for informing treatment decisions for patients. Most patients with glioblastoma are now asking, "If I'm not methylated, what clinical trial can I join?" The moderator asked the panelists to consider whether FUS liquid biopsy could adequately detect methylation levels and whether that could change patient care. Could 1p/19q co-deletion and other important markers also be detected? Key discussion points included the following:

High level Discovery

- Even before considering FUS, standards must be determined. It would be useful to have a biomarker panel or approach that considers CNS-specific signatures in the blood and detection of changes to this signature, possibly coming from a tumor, compared to normal. A multi-analyte approach would be great and provide much more robust information but would need to be standardized. The clinical significance would lie in determining whether a tumor or mutational repertoire is present.
- Consider the influence of FUS itself on the tumor landscape, especially proteomics. There are many confounders to consider. Preclinical testing is confounded by operator-specific technical variables such as anesthesia and microbubble parameters.
- Standardize protocol, aggregate data, and ensure it is normalized across different machines; then, analytical validation and reproducibility are needed. Standardization of total mechanical energy deposited, volume, etc. is needed. Need to standardize preanalytical variables and outcome assessment.

- To understand FUS's contribution to variability, assays done before and after FUS need to be compared. The standards still need to be determined.
- A major strength of FUS is its ability to be noninvasive and spatially selective. FUS can target active enhancing tumors, infiltrative edges, and leading margins, and the signatures will likely be different (genetic alterations, immune cells, normal neural cells interacting with tumors, etc.) in the various regions.
- A positive control in sampling potential progression vs pseudoprogression is needed.

Methylation

- Achieving the needed level of methylation detail with liquid biopsy would involve many variables and require a large amount of data and samples.
- Studying targeted areas with MGMT methylation is easier than studying the genome wide classification with thousands of different subtypes. Detecting MGMT methylation in blood and correlating it with the tumor is challenging, but researchers have measured MGMT status in the cerebrospinal fluid (CSF) and found a 75% to 80% correlation with tumor tissue methylation status. Most institutions will do MGMT analysis based on tissue received from surgery, and results could take weeks to receive and affect clinical trial enrollment. A library of DNA methylation fingerprints is first created, and then from that library, it is possible to query the MGMT locus. Using blood to detect methylation status is challenging as the amount of coverage over any specific locus is small, so to detect deletions needs more coverage—having an enriched template count (with FUS) would help.
- There is currently no data to suggest that following MGMT methylation over time is important for making decisions about clinical management, only to help with initial resection. Window of opportunity for using liquid biopsy MGMT may be restricted to inoperable cases.
- There is a new 6-day methylation cfDNA test for MGMT status and somatic variants. A test at NYU introduced MRD testing on WGS by measuring CNV across the genome at very low LOD and using AI.

Specific Analytes

- Chromosome 7 gain and 10 loss is a good biomarker, but its sensitivity and specificity need boosting. Can weight LB results and copy number changes so even if a small amount of loss 10 gain 7 is detected, this is confirmation of a GBM as this is never seen in healthy brain tissue.
- Regarding biomarkers and immunotherapies, some recent early work is looking at extracellular RNA signatures. Questions remain about whether analytes at the DNA level are appropriate to examine for prognostication versus diagnostic

applications. There is strong evidence in the literature for exosomal PD-L1 as a biomarker. The ability to monitor a peripheral biomarker of intratumoral PD-L1 expression could be useful for surveillance.

Cerebrospinal Fluid Analysis

- It would be interesting to study how FUS alters analytes in the CSF.
- Dr. Hong Chen described preliminary data in mice DIPG models before and after FUS in plasma and CSF. Enriched EGFR RNA was found in plasma, and enriched EGFR DNA was found in CSF.
- Dr. Leonara Balaj tested EGFRvIII in CSF and blood with longitudinal samples. It can detect mutations in both and may be able to monitor disease (in press). It takes 24 hours for the signal to correlate with CSF and a bit later to dump into blood.

Other Methods to Assess Treatment Effects

- Other useful technologies for assessing treatment effects include Amino acid PET or hydrochloride carbon- 13 as a measure of metabolism. 85% accuracy for GBM.
- RANO histopathology standardization for GBM is currently quite poor and does not even include molecular profiling and may not even correlate with clinical outcomes.
- Perhaps adjust the dose of therapeutics based on markers for hypoxia or use AA PET to define the target and change the dose.

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Reference

1 Lo YMD, Han DSC, Jiang P, Chiu RWK. Epigenetics, fragmentomics, and topology of cell-free DNA in liquid biopsies. *Science*. Apr 9 2021;372(6538)doi:10.1126/science.aaw3616

Key Clinical Needs

Burning Question 10

What are the key clinical needs that could be addressed with LB, and how does this fit with clinical workflow as opposed to other methods (CSF sampling without FUS)?

Moderator
Susan Chang

Panelists
Manmeet Ahluwalia, Nino Chiocca, Michael Vogelbaum, Patrick Wen

When considering a trial of a potential biomarker and liquid biopsy, researchers need to address several factors, including the biological rationale for the use of the biomarker, the measurement methods and assay considerations, the understanding of potential variables with FUS parameters, how to characterize the relationship between the biomarker and the outcome of interest, and the treatment required for the proposed context of use (where applicable). Researchers must determine the type of data needed to assess the strength of association between the biomarker and its proposed outcome (e.g., retrospective, or prospective, registry data, and/or randomized controlled trial (RCT) data), the reproducibility of the supportive data, and the statistical methods necessary to demonstrate the hypothesized relationships for the context of use.

Potential clinical settings for the use of focused ultrasound-enhanced liquid biopsies for GBM include:[1]

Susceptibility/risk
To indicate the potential for developing a tumor.

Diagnostic
To detect or confirm the presence of a tumor and augment detection of tumor-specific elements.

Monitoring tumor burden
Recurrence and pseudoprogression

Prognostic
To identify the likelihood of a clinical event.

Predictive
To identify individuals who are likely to experience a favorable or unfavorable outcome and help with treatment selection.

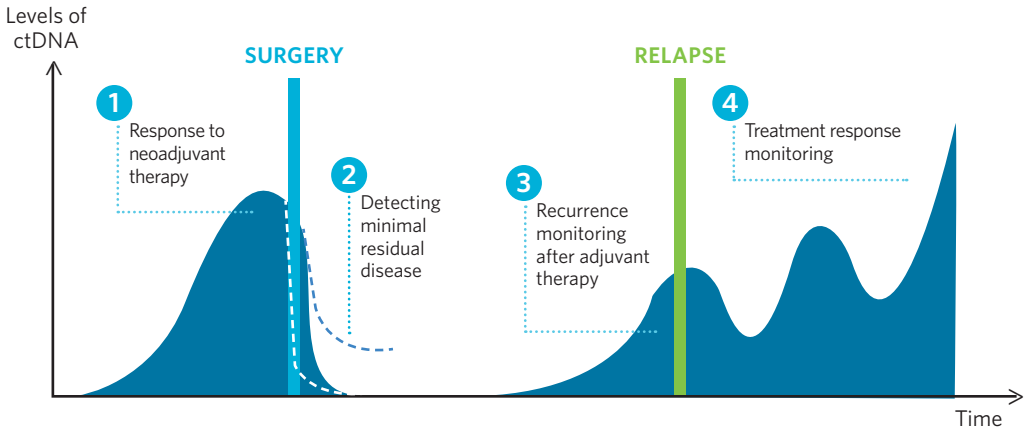
Figure 2

Key clinical needs potentially addressed by LB

What are the key clinical nees that could be address with LB, and how does this fit with clinical workflow as opposed to other methods (DSF sampling without FUS)?

Key clnical needs:

- Susceptibility/risk
- Diagnostic
- Monitoring tumor burden–recurrence and pseudo progression
- Prognostic
- Predictive–treatment selection
- Pharmacodynamic/response–precision medicine
- Safety



Pharmacodynamic/response

Precision medicine, Window of Opportunity trials: to show that a biological response has occurred and can serve as a surrogate of efficacy.

Safety

To indicate the likelihood, presence, or extent of toxicity and serve as a surrogate of long-term safety for the patient.

The panelists were asked to comment on the key clinical needs that could be addressed with liquid biopsy and discuss how this approach could fit with the clinical workflow when compared with other methods. How could liquid biopsies be used when thinking about biomarkers in general?

Diagnostic

- Useful for inoperable tumors or as a noninvasive method for characterization. Having specific biomarkers for disease-modifying alterations, such as isocitrate dehydrogenase 1 (IDH1) mutations, could be very helpful. If the IDH1 assay could be validated and have a higher sensitivity than 80%, it would support the value of combining with FUS.
- A needle biopsy can be difficult for some patients, especially children as well as some adults. Liquid biopsy may be a particularly useful approach for diffuse intrinsic pontine gliomas (DIPGs) for HK327 so patients can enroll in a clinical trial.
- Consider that liquid biopsy may play a larger role in tracking the course of the tumor and resistance and a lesser role in initial diagnosis.
- Even though needle biopsies in most patients are possible, they still come with risks, and thus, tissue biopsy and LB could be complementary approaches, or one or the other.

Monitoring Tumor Burden and Pseudoprogression

- A systematized approach that involves the knowledge base of surgeons, pathologists, and radiologists, along with registering tissues, doing biopsies, and marrying the findings to liquid biopsies, may be the best way to handle this complicated, multi-component effort. Another challenge is the need to get baseline samples. The RANO recurrence group is working to standardize pathology reads for residual vs. recurrent tumors.
- Either a baseline pre- and post-FUS LB before surgery (some patients come for surgery without FUS) or multiple time points after surgery (as a new baseline) are needed to note trends—increasing levels suggest recurrence. Obtain samples serially with MRI. The volume of tissue sonicated with FUS is probably important to standardize.
- Using a marker as an indicator of tumor burden can raise questions about its sensitivity and whether the marker is really a true surrogate of tumor burden or of something released due to brain injury during surgery. Would the liquid biopsy represent 1cc or 5-10 cc of recurrent tumor?
- Moving the field of liquid biopsy forward will require studies of prospectively acquired cohorts of patients in randomized control trials. Please note: Increasing allele frequencies correlate with worse survival and the volume of tumor correlates with LB levels. Initially, a negative LB is likely related to low detection limit, but once the patient starts therapy with chemoradiation, lots of cells die, releasing material so the LB becomes positive and thus may only be able to detect changes after treatment (Current study is following 8 patients at multiple time points in the study with IDH-1. These findings were reproducible within a standard deviation when taking the same sample and testing it twice for validation).

Predictive

- LB could be used to monitor treatment; for example, is the patient responding to immunotherapy with increased drug delivery after FUS? Can we detect it earlier than MRI? Is that predictive biomarker based on tissue specimens?
- Targeted therapies exist for BRAF mutations, with 1/3 of patients responding. Consider possibly partnering with these Phase 2 studies. Monitor that mutation over the course of therapy, gauge the drug's target engagement, and show eligibility for clinical trials.

Pharmacodynamic Response for WoO Studies/Other Comments

- Whether a liquid biopsy biomarker could serve as a surrogate of response to therapy depends on how tightly the marker is linked to the treatment and its reliability as an indicator of success or failure. This question is appropriate for preclinical exploration and validation. Rather than only using FUS to increase drug delivery, a potential use may be to track what happens with the increased release of a biomarker representative of target engagement. Consider transcriptomics for analysis.
- Even though a drug is given orally every day, it may only need FUS once a week to increase drug concentration and prime the tumor microenvironment. Then, target engagement can be monitored as well.
- Can potentially enhance the presence of ctDNA in the blood by increasing analyte quantity with FUS or radiation or by stabilizing existing ctDNA particles with AB or nanoparticle
- The potential for using liquid biopsy to eliminate the need for an MRI may be a big win for communities where MRI is not accessible.

Safety

- What is the meaning of T2* on MRI, and is there a better LB marker for safety and potentially cognitive risk?
- Leveraging learning across disease states is important. Biomarkers that give information about toxicity and efficacy simultaneously would be useful. Early work indicates that methylation may be a consideration.

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Reference

1 FDA-NIH Biomarker Working Group. *BEST (Biomarkers, endpoints, and other Tools) resource*. U.S. Food and Drug Administration (FDA)/National Institutes of Health (NIH); 2016:1 online resource (1 PDF file (iv, 11 pages)). https://www.ncbi.nlm.nih.gov/books/NBK326791/

Action Items & Considerations

Action Items

1. Form a working group to create guidelines and recommendations for FUS-enhanced LB which should include:
   a. How to collect samples
   b. Establishment of common language for data elements across platforms
   c. Technical and clinical context variables
   d. Normalization of biomarker analysis
2. Create reporting standards with FUS input parameters and output readings confirming BBBO.
3. Develop BBBO phantom to ensure parameters create a desired effect that is repeatable. Consider a phantom that can be used to detect focal pressures and report acoustic emissions feedback.
4. Advance knowledge of confirming BBBO beyond gadolinium enhancement with acoustic emissions, PET labeling, and WoO studies.
5. Encourage Dr. Konofagou to publish her results on the long-term outcomes of repeated FUS BBBO in non-human primates over 10 years, including cognitive and histologic outcomes.
6. Follow InSightec working group that will assess T2* changes after FUS BBBO.
7. Include patient-reported outcomes in clinical trials to identify barriers to clinical adoption.

Considerations

1. It is important to work with regulatory agencies to decrease barriers for studying drug–device combinations.
2. Amino acid PET imaging may improve FUS targeting for residual active disease.
3. Window of opportunity studies may provide insights into the effects of various FUS parameters and mechanisms of action (such as antigen presentation) on the tumor microenvironment.
4. Preclinical data, including biodistribution studies and efficacy, are needed for the FDA to guide practical decisions on whether to give the drug before, during, or after FUS BBBO.

5 Consider designing FUS LB clinical trials to address the following for brain tumors:

- **a.** Distinguishing progression versus pseudoprogression
- **b.** Utilizing LB as a diagnostic tool in the upfront setting for inclusion in clinical trials
- **c.** Monitoring response to a specific therapy
- **d.** Engaging in window of opportunity trials for drug delivery
- **e.** Using LB results for treatment adaptation, and tunability of the technology

6 Future FUS BBBO studies for LB should collect various analytes to create knowledge. Be thoughtful about study design based on the goal:

- **a.** Consider clinical stage of disease and related biomarkers
- **b.** Collect tumor-specific biomarkers or analytes to gauge response to treatment
- **c.** Study the interaction between tumor and the immune system
- **d.** Find analytes that measure safety and degree of BBBO

7 Compare results by using the same preclinical model across different machines (after standardization of protocols). Include details regarding the study design, such as how the tumors were implanted, and type and amount of anesthesia.

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Workshop Participants

Baptist Health South Florida
Manmeet Ahluwalia, MD, MBA
Deputy Director and Chief Scientific Officer

Brigham and Women's Hospital
Nathan McDannold, PhD
Research Director, FUS Program

Brown University
Clark Chen, PhD, MD
Professor

Carthera, SA
Michael Canney, PhD
Scientific Director

Columbia University
Elisa Konofagou, PhD
Professor

Dana-Farber Cancer Institute
Nino Chiocca, MD, PhD
Surgical Director

Patrick Wen, MD
Director, Neuro-Oncology Division

Denali Therapeutics & The University of Minnesota
Robert Thorne, PhD
Affiliated Pharmaceutics Faculty, Denali Fellow

Focused Ultrasound Foundation
Suzanne LeBlang, MD
Director, Clinical Relationships

Lauren Powlovich, MD
Associate Chief Medical Officer

Emily White, MD
Managing Director, Operations and FUS Partners

Georgia Institute of Technology
Costas Arvanitis, PhD
Assistant Professor

Harvard Medical School
Leonora Balaj, PhDD
Assistant Investigator, Assistant Professor

Johns Hopkins University School of Medicine
Chetan Bettegowda, PhD, MD
Professor

MD Anderson Cancer Center
Jason Huse, PhD, MD
Principal Investigator, Professor

Medical & Health Writing
Mary Love
Medical Writer

Moffitt Cancer Center
Michael Vogelbaum, MD, PhD
Chief of Neurosurgery

Quest Diagnostics Foundation, Inc.
James Godsey, PhD
Advanced Dx at R&D

The Sontag Innovation Fund
Scott Davis, PhD
Senior Associate

Hilary Keeley, JD
Executive Director

Sunnybrook Health Sciences Centre
Kullervo Hynynen, PhD
Director & Senior Scientist, Imaging Research

United State FDA Center for Devices & Radiological Health
Subha Maruvada, PhD
Acoustics Engineer

University of California, San Francisco
Susan Chang, MD
Director

Kazim Narsinh, MD
Interventional Neuroradiologist

University of Maryland School of Medicine
Graeme Woodworth, MD, FACS
Professorr

University of Pennsylvania Health System
Seyed Ali Nabavizadeh, MD
Assistant Professor, Radiology

University of Pittsburgh
Samuel Sprowls, PhD
Postdoctoral Research Associate

University of Virginia
Natasha Sheybani, PhD
Assistant Professor

Washington University in St. Louis
Hong Chen, PhD
Assistant Professor

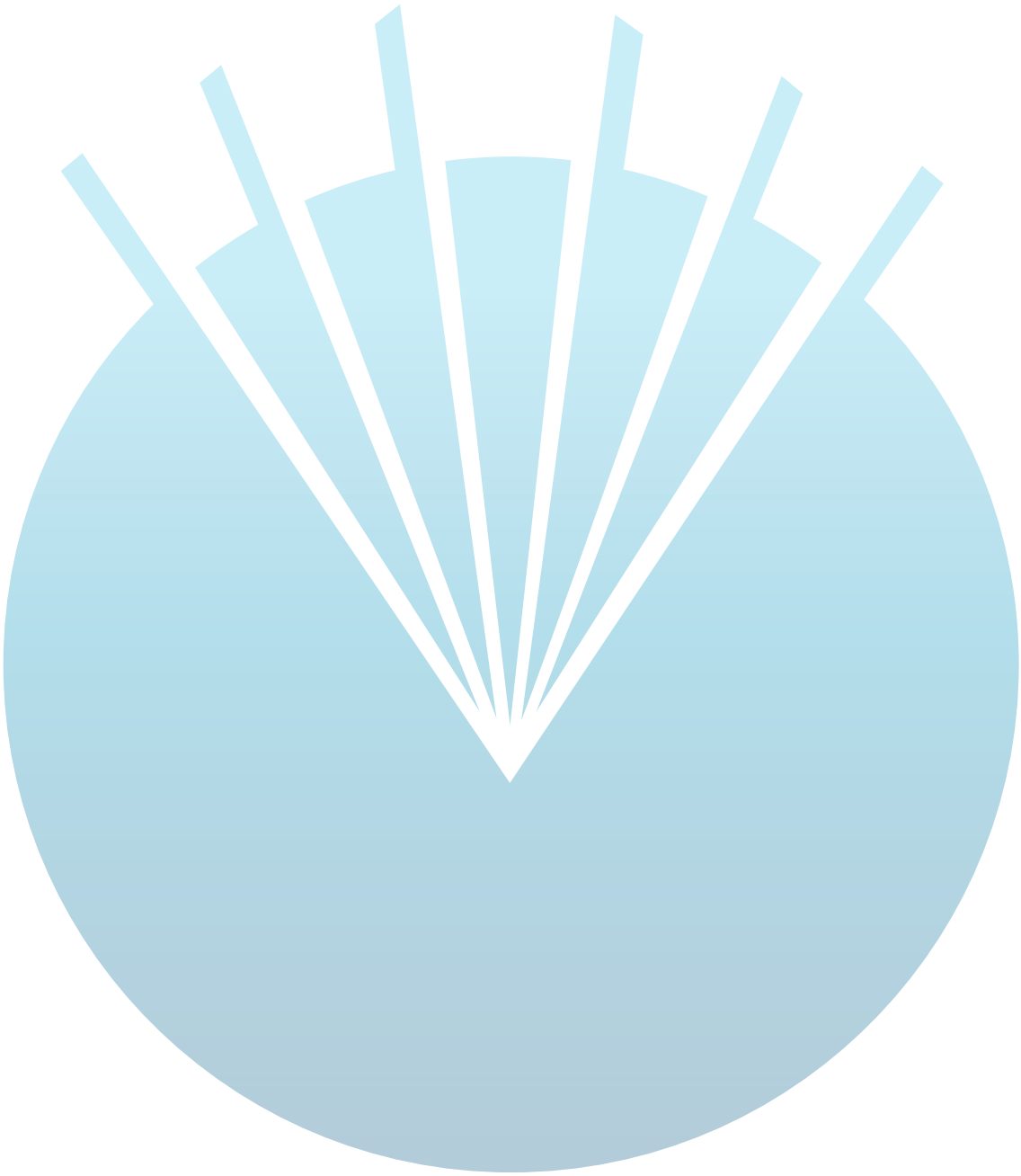
Supplemental Information

In advance of the meeting, a recommended list of relevant research articles was provided to the participants and can be accessed at: Preparatory reading materials.

Acknowledgements

This workshop was sponsored jointly by the Focused Ultrasound Foundation and The Sontag Foundation. We sincerely thank the steering committee for planning and creating the program and the burning questions: Graeme Woodworth, Costas Arvanitis, Susan Chang, Chetan Bettegowda, Hilary Keeley, Scott Davis, Suzanne Leblang, Lauren Powlovich, and Emily White.

Suzanne Leblang and Lauren Powlovich wrote this summary, which the steering committee approved and edited. Mary Love, medical writer for Orvos Communications, provided a draft transcript of the workshop.



FOCUSED ULTRASOUND FOUNDATION

1230 Cedars Court, Suite 206
Charlottesville, VA 22903

fusfoundation.org

THE SONTAG FOUNDATION

816 A1A North, Suite 201
Ponte Vedra Beach, FL 32082

sontagfoundation.org