

23 9. Department of Computer Science and Engineering, Washington University in St. Louis, Saint
24 Louis, MO 63130, USA

25 10. Siteman Cancer Center, Washington University School of Medicine, St. Louis, MO, 63110,
26 USA

27 11. Division of Neurotechnology, Department of Neurosurgery, Washington University School of
28 Medicine, Saint Louis, MO, 63110, USA

29 12. Department of Neuroscience, Washington University School of Medicine, Saint Louis, MO,
30 63110, USA

31 13. Center for Innovation in Neuroscience and Technology, Washington University School of
32 Medicine, Saint Louis, MO, 63110, USA

33 14. Department of Mechanical Engineering and Materials Science, Washington University in St.
34 Louis, Saint Louis, MO 63130, USA

35

36 # These authors contributed equally to the study.

37 * ECL and HC are co-senior authors.

38 **ABSTRACT**

39 Sonobiopsy is an emerging technology that combines focused ultrasound (FUS) with
40 microbubbles to enrich circulating brain disease-specific biomarkers for noninvasive molecular
41 diagnosis of brain diseases. Here, we report the first-in-human prospective trial of sonobiopsy in
42 glioblastoma patients to evaluate its feasibility and safety in enriching circulating tumor biomarkers.
43 A nimble FUS device integrated with a clinical neuronavigation system was used to perform
44 sonobiopsy following an established clinical workflow for neuronavigation. Analysis of blood
45 samples collected before and after FUS sonication showed enhanced plasma circulating tumor
46 biomarker levels. Histological analysis of surgically resected tumors confirmed the safety of the
47 procedure. Transcriptome analysis of sonicated and unsonicated tumor tissues found that FUS
48 sonication modulated cell physical structure-related genes but evoked minimal inflammatory
49 response. These feasibility and safety data support the continued investigation of sonobiopsy for
50 noninvasive molecular diagnosis of brain diseases.

51

52 INTRODUCTION

53 The diagnostic evaluation of glioblastoma (GBM) relies on neuroimaging by magnetic resonance
54 imaging (MRI) and computed tomography, followed by surgical resection or biopsy for histological
55 confirmation and genetic characterization. Alternative approaches to obtain information on a brain
56 lesion without surgery include lumbar puncture and blood draw¹. Lumbar puncture for cerebral
57 spinal fluid-based liquid biopsy is uncomfortable and carries procedural risk, limiting its use for
58 repeated testing. In contrast, blood-based liquid biopsy is a noninvasive, rapid, and inexpensive
59 method to obtain highly relevant information about the tumor². This approach detects circulating
60 tumor-derived biomarkers, such as DNA, RNA, proteins, and extracellular vesicles shed by tumor
61 cells. It is a promising approach for the diagnosis, molecular characterization, and monitoring of
62 brain tumors³. Although blood-based liquid biopsy-guided personalized therapy has already
63 entered clinical practice to treat several cancers^{4,5}, extending it to brain cancer remains
64 challenging⁶. Brain tumor-derived circulating tumor biomarkers are generally detected only at low
65 abundance and in a limited number of patients, which makes analysis difficult in routine clinical
66 practice⁷⁻¹⁰. This low abundance is primarily due to the blood-brain barrier (BBB), a physical
67 barrier that prevents the transfer of brain tumor biomarkers into the peripheral circulation, resulting
68 in low test sensitivity^{6,11}. Even when the BBB is disrupted in GBM, the release of tumor-specific
69 biomarkers into the peripheral circulation remains limited¹.

70 Transcranial low-intensity focused ultrasound (FUS) in combination with intravenously injected
71 microbubbles is a promising technique for noninvasive, spatially targeted, and reversible
72 disruption of the BBB¹². FUS can penetrate the skull noninvasively and focus on virtually any brain
73 region with millimeter-scale accuracy. Microbubbles, traditionally used as blood-pool contrast
74 agents for ultrasound imaging, amplify and localize FUS-mediated mechanical effects on the
75 vasculature via FUS-induced cavitation (i.e., microbubble expansion, contraction, and collapse).
76 Microbubble cavitation generates mechanical forces on the vasculature¹³ and reversibly increases

77 the BBB permeability in the FUS-targeted brain region. Typically, the permeabilized BBB usually
78 reseals after a few hours¹⁴. Recent clinical studies have demonstrated the feasibility and safety
79 of FUS-mediated BBB disruption for brain drug delivery in patients with glioblastoma^{15–20},
80 Alzheimer's disease²¹, amyotrophic lateral sclerosis²², and Parkinson's disease²³

81 We hypothesized that FUS-induced BBB disruption enables "two-way trafficking" between the
82 brain and bloodstream²⁴. With FUS-mediated BBB disruption, circulating agents can enter the
83 brain, while brain tumor-derived biomarkers can be released into the bloodstream for potential
84 diagnostic access. We term this FUS-induced release of biomarkers into the blood circulation for
85 blood-based liquid biopsy as sonobiopsy. Sonobiopsy disrupts the BBB at the spatially targeted
86 brain location, releases tumor-derived biomarkers from precisely defined tumor locations into the
87 blood circulation, and enables timely detection of biomarkers in the blood to minimize clearance.
88 Our previous study provided compelling preclinical evidence that sonobiopsy enriched circulating
89 RNA, DNA, and proteins in small and large animal models^{24–28}. Recently, we found that
90 sonobiopsy improved the detection sensitivity of GBM-specific EGFRvIII mutation from 7.14% to
91 64.71% and TERT C228T from 14.29% to 45.83% in a mouse GBM model. It also improved the
92 diagnostic sensitivity of EGFRvIII from 28.57% to 100% and TERT C228T from 42.86% to 71.43%
93 in a porcine GBM model²⁹. By retrospectively analyzing blood samples collected from FUS-
94 mediated drug delivery clinical trials, Meng et al. provided preliminary clinical evidence that FUS-
95 induced BBB disruption increased the concentrations of circulating biomarkers, including cell-free
96 DNA, neuron-derived extracellular vesicles, and brain-specific protein³⁰.

97 The most widely used FUS device in current brain drug delivery clinical trials is the MRI-guided
98 FUS system, ExAblate Neuro, from InSightec Inc. This system utilizes a hemispherical-shaped
99 FUS transducer with 1,024 elements and an aperture of 30 cm³¹. It was initially designed for
100 thermal ablation and has been approved by the United States Food and Drug Administration to
101 treat essential tremors. While this device can be adapted for sonobiopsy, it is expensive (>\$3M)

102 and requires MR-compatible hardware, MR scanner time, and extensive training to operate the
103 device. Although neuronavigation-guided FUS devices were developed for drug delivery, they
104 need a robotic arm for positioning heavy FUS transducers with large apertures and customized
105 optical trackers to guide the positioning of the transducer. FUS devices used for drug delivery
106 require high spatial precision and a large treatment volume to deliver drugs to cover the whole
107 diseased brain region efficiently. However, FUS devices for sonobiopsy do not need to deliver
108 therapeutic drugs or cover the entire tumor. Affordable and easy-to-use FUS devices are needed
109 for sonobiopsy to target specific regions inside the tumor for spatially targeted biomarker release.

110 Here, we present a small-aperture FUS device that is nimble and easily integrated with existing
111 clinical neuronavigation systems. This device enables the sonobiopsy procedure to be performed
112 using a clinical workflow similar to existing neuronavigation-guided tissue biopsy. A pilot
113 prospective sonobiopsy clinical study was conducted on GBM patients using this device to
114 evaluate the feasibility and safety of sonobiopsy. Our results demonstrate that sonobiopsy
115 enriched circulating GBM-specific biomarkers without causing any evident tissue damage.

116

117 RESULTS

118 Study patients

119 The aim of this prospective single-arm trial was to assess the feasibility and safety of sonobiopsy
120 in patients with GBM. The trial was approved by the Washington University in St. Louis Institute
121 Review Board and registered with clinicaltrials.gov (Identifier: NCT05281731). Written informed
122 consent was obtained from all participants prior to study enrollment. Patients with a lesion in the
123 brain with imaging characteristics consistent with GBM were screened for the clinical trial. Of the
124 five patients screened for the study, three patients (two men and one woman; median age 65
125 years; range 58–74 years) met the inclusion/exclusion criteria and were enrolled in the trial (**Table**
126 **1, Fig. 1a**). Details of the inclusion and exclusion criteria are provided in Supplementary Table
127 S1. The primary outcome of this study was to evaluate the feasibility of sonobiopsy to increase
128 GBM tumor-specific biomarker levels in the post-sonication blood samples compared with pre-
129 sonication blood samples. The secondary study outcome was to verify that there was no evidence
130 of brain tissue injury associated with the procedure.

131 Sonobiopsy procedure was successful

132 Sonobiopsy was performed after the patients were prepared for the surgery in the operating room
133 and before the planned surgical removal of the GBM tumor. Patients were under general
134 anesthesia, and vital signs were continuously monitored by an anesthesiologist. Sonobiopsy was
135 performed using a neuronavigation-guided FUS transducer (**Fig. 1b**). The timeline of the
136 procedure is illustrated in **Fig. 1c**. MRI and CT images acquired before the procedure were loaded
137 in the neuronavigation system (Stealth S8, Medtronic) and used for spatial registration of the
138 patient's head position. The patient's hair above the tumor region was shaved. Degassed
139 ultrasound gel was applied to the cleaned scalp for acoustic coupling. The FUS transducer with
140 a water bladder attached was coupled to a standard neuronavigation probe with a customized

141 adaptor (**Fig. 1c**). The focus position of the FUS transducer was calibrated beforehand to be 80
142 mm from the tip of the stereotactic probe. An 80 mm offset was added in the neuronavigation
143 software so that the tip of the "virtual probe" indicated the location of the FUS focus (**Fig. 2a**). The
144 FUS transducer was mechanically positioned to align its focus at the planned tumor location. The
145 acoustic pressure field was simulated based on the final trajectory of the probe, and the skull
146 attenuation was estimated based on the simulation (**Fig. 2b**). The offset between the planned
147 target and the actual target based on the simulation was found to be 1.91 ± 0.97 mm in lateral
148 direction and 5.29 ± 0.83 mm in the axial direction. The acoustic output pressure of the FUS
149 transducer was adjusted to control the mechanical index (in situ acoustic pressure/square root of
150 frequency) to be within 0.4–0.8 (**Table 2**). Microbubbles (Definity, 10 μ L/kg) were intravenously
151 injected by an anesthesiologist, followed by FUS sonication for 3 mins.

152 The FUS transducer had an acoustic sensor inserted in its center. The sensor had three functions:
153 quality assurance to ensure the FUS transducer had consistent output before the procedure (**Fig.**
154 **S1a, S1b**), acoustic coupling quality assessment by performing cavitation detection during FUS
155 sonication before microbubble injection (**Fig. S1c, S1d**), and FUS treatment monitoring after
156 microbubbles were injected (**Fig. 2c, 2d**). Real-time cavitation monitoring provided an effective
157 tool for monitoring the treatment procedure. The stable and inertial cavitation levels were
158 quantified based on the frequency spectrum of the acquired signals to quantify the bubble activity
159 under stable oscillation (stable cavitation generates harmonic signals) and violent collapse
160 (inertial cavitation generates broadband signals). The cavitation doses calculated by integrating
161 the cavitation level over time for all three patients are summarized in **Table 2**.

162 Blood samples were collected immediately before (5 mins pre-FUS) and at different time points
163 post-sonication. After the last blood collection, surgery was performed, and tumor tissue samples
164 were collected from the FUS sonicated and nonsonicated tumor regions under the

165 neuronavigation guidance. The total procedure time from when the patient was prepared and
166 ready for the sonobiopsy procedure to the end of FUS sonication was 22.7 ± 6.6 min.

167 **Sonobiopsy enriched circulating GBM-specific biomarkers**

168 To evaluate the feasibility of sonobiopsy in enriching circulating GBM biomarkers, we first
169 quantified plasma levels of glial fibrillary acidic protein (GFAP), a reliable and commonly used
170 liquid biomarker for GBM³². Using ultrasensitive single-molecule array (Simoa) assay, we found
171 that sonobiopsy increased plasma GFAP levels for all three patients (**Fig. 3a**), with the maximum
172 increase being 1.2-fold for G02 at 30 mins post-FUS, from 15.6 ± 0.83 ng/mL to 19.3 ± 2.4 ng/mL
173 of plasma ($p < 0.05$). We further analyzed the total cell-free DNA (cfDNA) levels in the plasma (**Fig.**
174 **S2**). We found that sonobiopsy significantly increased the concentration of mono-nucleosomal
175 cfDNA fragment (120–280 bp) for all time points post-FUS, except at 5 mins for G01 (**Fig. 3b**).
176 The maximum increase was 1.6-fold for G02 at 30 mins, from 30.3 ± 4.2 ng/mL to 63.9 ± 4.6
177 ng/mL of plasma ($p < 0.0001$).

178 To assess the potential of sonobiopsy to improve the detection of patient-specific tumor variants
179 in the plasma, we utilized a personalized tumor-informed ctDNA assay (Invitae Personalized
180 Cancer Monitoring assay). This assay involved performing whole exome sequencing (WES) on
181 the tumor and normal tissue. Sequencing results allowed the identification and selection of up to
182 50 clonal, somatic, single-nucleotide variants present in the tumors but not the matched normal
183 samples. The selected single-nucleotide variants were used in the design of a patient-specific
184 panel, which was used to detect circulating tumor DNA (ctDNA) in patient plasma samples. We
185 compared the absolute level of patient-specific tumor variants in the plasma samples collected
186 before and after FUS using tumor variant copies/ml plasma. Our results indicated that sonobiopsy
187 successfully enhanced the detection of patient-specific tumor variants in the plasma of G02 and
188 G03 (**Fig. 3c**). For these two patients, the kinetics of biomarker changes following sonication

189 revealed that higher tumor variant copies were detected at later time points. Specifically, G02
190 reached the highest number of tumor variant copies at 30 min post-FUS, compared with pre-FUS
191 (5.6 vs. 2.9 for post vs. pre-FUS, $p < 0.001$). For G03, the final blood sample was acquired at 10
192 mins post-FUS, and the tumor variant copies were the highest at this time point compared with
193 pre-FUS (4.2 vs. 3.1 for post vs. pre-FUS, $p < 0.01$). However, no clear increase in tumor variant
194 copies was observed for G01.

195 A combination of TERT promoter mutation and IDH wild type is the most common genotype
196 observed in GBM. TERT promoter mutations are present in 62% of GBM patients and associated
197 with poor treatment outcome^{33,34}, while IDH1 mutations are also important diagnostic and
198 prognostic markers for glioma³⁵. To further investigate the potential of sonobiopsy in discerning
199 these two mutations, we analyzed the amount of TERT mutation (C228T and C250T) and IDH1
200 mutation (R132H) in the plasmas with digital droplet PCR (ddPCR). ddPCR analysis on tumor
201 tissue samples found that all three patients were positive for TERT mutations but IDH1 wild type
202 (**Fig. S3**). IDH1 mutation (R132H) was consistently undetectable in any of the sonobiopsy plasma
203 samples. In contrast, TERT mutations (C228T and C2250T) were detected at higher levels by
204 sonobiopsy than with conventional blood draw pre-FUS, with 1.8 fold increase for G01 at 30 mins
205 post-FUS, 4.3-fold increase for G02 at 30 mins post-FUS, and 3.0-fold increase for G03 at 10
206 mins post-FUS (**Fig. 3d**).

207 **Sonobiopsy did not induce detectable tissue damage**

208 During the FUS sonication procedure, we did not observe any significant fluctuations in vital signs,
209 such as heart rate and respiration, nor did we note any adverse events. Following FUS sonication,
210 we observed no evidence of hemorrhage on the surface of the brain due to FUS sonication (**Fig.**
211 **4a**). Additionally, tissue samples obtained from both the sonicated and nonsonicated tumor
212 regions after tumor dissection were stained by hematoxylin and eosin (**Fig. 4b**). Signs of tissue
213 damage, including microhemorrhages and other cytoarchitectural changes, were not detected.

214 **Sonobiopsy did not induce evident inflammation/immune responses**

215 To provide a comprehensive safety profile of FUS sonication on the tumor, we conducted
216 transcriptome analysis of sonicated and nonsonicated tumor tissues. We identified differentially
217 expressed genes (DEGs) using hierarchical clustering analysis following strict criteria of the
218 absolute value of \log_2 (fold-change) > 2 and Pvalue < 0.05 (**Fig. 5a**). Our analysis identified 34
219 DEGs out of the total 17,982 genes (0.19%), among which 19 transcripts were identified as
220 upregulated DEGs associated with sonication, while 15 were downregulated DEGs (**Fig. 5b**).

221 The gene ontology (GO) analysis of the upregulated and downregulated DEGs showed that the
222 enriched GO terms were related to the physical structures of cells, including their interactions with
223 neighboring cells and their surrounding extracellular matrix (**Fig. 5c**). This suggests that FUS-
224 combined with microbubbles caused mechanical perturbation to the cell-cell and cell-matrix
225 interaction. The genes related to cell physical structure that were upregulated and downregulated
226 are further summarized in **Fig. 5d**. Real-time qRT-PCR analysis was performed and verified the
227 upregulation and downregulation of these genes (**Fig. S4**).

228 FUS with microbubbles was reported by previous studies to induce sterile inflammation in healthy
229 mouse brains^{36–38}. However, our analysis only identified one immune-related GO term, "immune
230 receptor activity," among the top 26 enriched GO terms (**Fig. 5e**). Further analysis of potential
231 inflammatory-immune-related GO terms involving at least one DEG identified only one significant
232 GO term (GO0140375, "immune receptor activity") that had more than one overlapped DEGs.
233 Among all the discovered GO terms, CX3CR1 and HLA-DQB2 were identified as upregulated
234 DEGs, and MARCO was identified as a downregulated DEG. The upregulation of CX3CR1 and
235 downregulation of MARCO were further confirmed by real-time qRT-PCR results (**Fig. S4**). Our
236 results suggest that the FUS procedure did not induce severe immune or inflammation responses.

237 **DISCUSSION**

238 We present the first prospective clinical study of sonobiopsy in GBM patients. Our study utilized
239 the nimble sonobiopsy device, which was seamlessly integrated with an existing clinical
240 neuronavigation system. The sonobiopsy procedure was performed using an established clinical
241 workflow for neuronavigation. Our findings provide crucial initial evidence that sonobiopsy can
242 enrich GBM biomarkers in the blood by targeting specific tumor locations and coordinating the
243 blood collection time.

244 We demonstrated significant technological advancements that the sonobiopsy device offers to
245 adopt this innovative technique in the clinic. First, the nimble design of the FUS device allows for
246 direct attachment of the FUS device to existing neuronavigation probe used by any clinical
247 neuronavigation system, enabling precise positioning of the FUS transducer with high accuracy.
248 Furthermore, this unique design also allows for easy integration of sonobiopsy into the existing
249 clinical workflow, eliminating the need for additional training of neurosurgeons to perform the
250 sonobiopsy procedure. This will reduce the barrier to adopting this technique in the future.
251 Importantly for future considerations, an operating room is not required. While cranial fixation and
252 anesthesia were used for the patients described in this work, it is not essential. Standard
253 navigation techniques that can be used without fixation and anesthesia could enable sonobiopsies
254 to be performed outside classic operative and procedural environments (e.g., hospital rooms and
255 clinics). Second, the sonobiopsy device also incorporates numerical simulation of the acoustic
256 energy delivered into the brain. This simulation provides critical guidance for the selection of
257 ultrasound parameters and allows visualization of the ultrasound beam shape and location inside
258 the brain. Third, the integration of the FUS device with cavitation detection enables the
259 development of quality assurance for the FUS device and real-time monitoring for the treatment.
260 Future work can integrate cavitation feedback control algorithms to regulate the FUS acoustic

261 pressure in real-time^{39,40}, which would ensure precise and consistent delivery of the ultrasound
262 energy.

263 We also demonstrated that sonobiopsy could be integrated with advanced blood-based biomarker
264 analysis techniques for the noninvasive and spatially targeted molecular diagnosis of GBM
265 without causing brain damage. ctDNA-based sequencing assays can be divided into two classes:
266 tumor-naïve assays and tumor-informed assays. Tumor-naïve assays use broad panel-based
267 sequencing assays for genotyping or tumor early detection with a detection limit of about 0.2%⁴¹;
268 Tumor-informed assays are designed in reference to mutations known from the tumor and can
269 reach a limit of detection as low as 0.01% variant allele frequency⁴¹. Examples of tumor-informed
270 assays include CAPP-Seq⁴², PhasED-seq⁴³, and personalized tumor-specific sequencing⁴³. In
271 this study, we used the Invitae Personalized Cancer Monitoring assay, which was developed to
272 detect residual molecular diseases by providing sensitive detection of ctDNA in the plasma
273 samples. Our results show that sonobiopsy significantly increased the detection of tumor variants
274 in two out of three patients. Given that this is the first prospective trial, the observation that 2/3 of
275 patients had a significant increase in the detected tumor variants suggests that sonobiopsy is a
276 promising technique for enriching plasma ctDNA. In addition to sequencing-based assays, ddPCR
277 is a targeted approach for rapidly detecting specific known mutations with high sensitivity and
278 tissue concordance^{44–46}. Thus, ddPCR was used in our study to detect ctDNA with prior
279 knowledge of the mutations expressed by the GBM tumors. The GBM tumors are known to have
280 TERT mutation but no IDH1 mutation. The ddPCR results demonstrate that sonobiopsy enriched
281 the level of TERT mutation without affecting the amount of IDH1 mutation, implying that
282 sonobiopsy can improve the sensitivity and specificity in mutation detection. The kinetics of
283 biomarker changes post-FUS indicate a trend of increasing ctDNA levels within the 5–30 min time
284 frame. Future studies are needed to determine the complete kinetics of biomarker release and
285 optimal blood collection time.

286 The prospective trial design implemented in this study allowed for the collection of GBM tissue
287 samples from the sonicated and nonsonicated tumor regions in each patient. This approach
288 provided an unprecedented opportunity to evaluate the bioeffects of FUS sonication on the tumor
289 in GBM patients. We conducted the transcriptome analysis of FUS effects on patient GBM tumors
290 obtained at 70.0 ± 6.1 minutes post-FUS sonication and observed only a minimal change of 0.19%
291 in gene expression after sonication. Most upregulated and downregulated genes were related to
292 the physical structures of cells, such as cell interactions with neighboring cells and the
293 extracellular matrix. This finding suggests that FUS-combined with microbubbles caused
294 mechanical perturbation to cell-cell and cell-matrix interactions. Notably, the downregulation of
295 MMP1 and MMP7 genes was previously reported to be associated with BBB integrity⁴⁷,
296 suggesting that the sonobiopsy procedure induced BBB disruption at the targeted tumor region.
297 FUS-induced BBB disruption has been shown to induce an inflammatory response in mice^{36–38},
298 but there have been no reports examining the immune response in GBM patients. Contrary to
299 these reports in mice, our study shows that only three genes CX3CR1, HLA-DQB2, and MARCO
300 were identified as DEGs related to the immune response in GBM tumors obtained at 70.0 ± 6.1
301 minutes post-FUS sonication. CX3CR1 and HLA-DQB2 were upregulated upon FUS sonication,
302 consistent with previously reported increased infiltration of CX3CR1-positive immune cells into
303 the tumor area after FUS-induced BBB disruption in mice⁴⁸. This lack of activation of the immune
304 response needs to be confirmed in the future using tissue samples acquired at later time points.

305 The results presented in this pilot clinical trial provide important insights into the potential for
306 sonobiopsy in noninvasive molecular characterization of GBM and other brain diseases (e.g.,
307 neurodegenerative diseases and psychiatric disorders). Sonobiopsy has the potential to achieve
308 several critical benefits after integration into clinical practice as a complement to neuroimaging
309 and tissue biopsy, including the identification of genetic features before surgical intervention,
310 enabling alterations in surgical strategy. It could also enable the rapid determination of the

311 molecular identity of suspicious lesions observed on neuroimaging scans, particularly in patients
312 who are poor surgical candidates. Furthermore, the ability to repeatedly sample and monitor
313 tumor recurrence and treatment response could provide valuable information to clinicians. In
314 challenging situations where assessment based on neuroimaging alone remains difficult, such as
315 distinguishing treatment-induced pseudoprogression from true relapse, sonobiopsy could provide
316 complementary information. Moreover, it has the potential to support investigations into tumor-
317 specific molecular mechanisms driving disease and accelerate the development of new treatment
318 strategies.

319 While this study presents milestone achievements in developing sonobiopsy for the molecular
320 diagnosis of GBM, several limitations exist. First, although the data were extremely promising to
321 show the feasibility and safety of sonobiopsy, this pilot study was performed with a small number
322 of GBM patients. Further studies with larger sample sizes are needed to confirm these initial
323 findings and establish the clinical utility of sonobiopsy. Second, we selected to target a single
324 brain location in this pilot study. The previous retrospective study by Meng et al. found that
325 increasing the sonication volume could increase the biomarker release efficiency³⁰. Future study
326 is needed to optimize the sonobiopsy procedure, including evaluating the impact of sonication
327 volume on the efficiency of sonobiopsy. Third, there is always a spatial shift in the brain during
328 the surgical dissection of the sonication brain tumor tissue. This potential shift could have
329 introduced an error in the localization of the FUS-sonicated tumor region. To reduce the potential
330 impact of this error, the targeted tumor region was selected to be located at the relative superficial
331 tumor location so that this region was encountered early in the surgery and before substantial
332 brain shift.

333 In conclusion, this study represents the seminal initial step in establishing the feasibility and safety
334 of sonobiopsy in the brain of patients with GBM. This technique enables noninvasive, spatially
335 targeted, temporally controlled detection of brain GBM biomarkers in the blood. The feasibility

336 and safety data obtained from this pilot study will enable the field to move forward in translating
337 sonobiopsy into impactful diagnostics for GBM and other brain diseases.

338 **METHODS**

339 **Study design**

340 This prospective, single-arm, single-center, first-in-human study was designed to evaluate the
341 feasibility and safety of sonobiopsy. This study was approved by the Research Ethics Board at
342 Washington University in St. Louis, School of Medicine, and was registered with ClinicalTrials.gov
343 (NCT0528173). All subjects provided written informed consent before enrollment. This trial
344 complied with the International Conference on Harmonization guideline for Good Clinical Practice,
345 Tri-Council Policy Statement on ethical conduct for research involving humans (TCPS-2).

346 **Sonobiopsy device**

347 A FUS transducer consisting of 15 concentric individual ring transducers with a center frequency
348 of 650 kHz (Imasonics, Voray-sur-l'Ognon, France) was used. The aperture of the transducer was
349 65 mm, and the focal distance was 65 mm (f-number = 1). The FUS transducer was integrated
350 with a passive acoustic detector at its center. The FUS transducer was driven by a commercial
351 FUS system (Image Guided Therapy, Pessac, France). The nimble FUS transducer was coupled
352 to the passive blunt probe (Stealth S8, Medtronic) through a customized adaptor. The adaptor
353 was attached to the back of the FUS transducer and connected to a cylinder that was aligned with
354 the central axis of the FUS transducer. The diameter of the cylinder matched that of the
355 neuronavigation probe. This adaptor design leveraged the light weight of the FUS transducer and
356 mechanically co-aligned the neuronavigation tracker to the central axis of the transducer. The
357 location of the FUS focus was calibrated to be 80 mm from the tip of the passive blunt probe along
358 the trajectory of the probe. An 80 mm offset was added in the neuronavigation software so that
359 the tip of the "virtual probe" indicated the location of the FUS focus.

360 **Sonobiopsy clinical workflow**

361 The overall workflow of this clinical study is summarized in **Fig. 1d**. It consists of four main steps:
362 treatment planning, patient preparation, FUS sonication, and blood and tissue collection.

363 Step 1: Treatment planning. CT and MRI images of the patient's head were acquired a few days
364 before the procedure. FUS sonication trajectory was planned using the Medtronic S8 planning
365 station (Medtronic Plc, Dublin, Ireland). The trajectory was selected using the following criteria:
366 close to 90° incident angle (best effort), focus depth below skin < 35 mm (limited by the focal
367 length of our FUS transducer), and avoiding ultrasound beam passing through the ear lobe and
368 eye. A full-wave acoustic simulation using the k-Wave toolbox was performed to estimate the
369 ultrasound pressure field distribution inside the brain and calculate the skull attenuation using
370 methods reported in our previous publication⁴⁹.

371 Step 2: Patient preparation. On the day of the procedure, Mayfield skull clamp (Integra
372 LifeSciences, Princeton, NJ, USA) was fixed to the patient's head under local and general
373 anesthetic. The skull clamp was then connected to the surgical table through a Mayfield bed
374 attachment. The stealth arc and Veltek arm were then connected, and the patient's head was
375 registered to the pre-acquired MRI/CT images. The planned FUS trajectory was then entered into
376 the neuronavigation system. A small patch of hair above the tumor region was shaved, and the
377 exposed skin was thoroughly cleaned with alcohol pads. Deionized water was filled into the
378 transducer water bladder, which was continuously degassed with a degassing system for more
379 than 15 min. Degassed ultrasound gel was applied liberally to the exposed skin area. FUS
380 transducer was then placed on the patient's head under the guidance of the neuronavigation
381 system.

382 Step 3: FUS sonication. The passive cavitation detector was used to check the quality of the
383 acoustic coupling between the FUS transducer and the skin. If broadband emissions were present
384 in the detect signals when the FUS was turned on without microbubble injection (**Fig. S1**), the
385 most likely cause was due to air bubbles trapped in the coupling media. In this case, we would
386 remove the FUS transducer, clean it, and re-apply the ultrasound gel. The input electrical power
387 was determined based on hydrophone calibration of the FUS transducer focal pressure over

388 different input electrical powers derated by the skull attenuation estimated in Step 1 based on k-
389 wave simulation. To ensure the safety of this study, the estimated in situ acoustic pressure was
390 selected to ensure the mechanical index (MI) was below 0.8, consistent with other FUS-BBBD
391 drug delivery clinical studies^{50,51}. Acoustic parameters besides input power were selected to be
392 the same as our previous preclinical work²⁹. The FUS parameters were: center frequency = 0.65
393 MHz (f_0); pulse repetition frequency = 1 Hz; pulse duration = 10 ms; treatment duration = 3
394 min. Fifteen seconds after FUS sonication began, microbubbles (Definity, Lantheus Medical
395 Imaging, North Billerica, MA) were administered intravenously by the standing anesthesiologist
396 at a dose of 10 μ L/kg body weight diluted with saline and followed with a saline flush. The injection
397 rate was controlled with the best effort to be 10 s/mL, recommended by the manufacturer. In
398 reference to our previous publication⁵², a custom MATLAB script was written to process the
399 acquired cavitation data to evaluate the stable cavitation and inertial cavitation levels. Briefly, the
400 stable and inertial cavitation levels were calculated as the root-mean-squared amplitudes of
401 subharmonic ($f_0/2 \pm 0.15$ MHz) and broadband (0.3–2 MHz after removing $f_0/2 \pm 0.15$ MHz and
402 $nf_0 \pm 0.15$ MHz where $n = 1, 2, 3$) signals, respectively.

403 Step 4: Blood and tissue collection. Blood samples (20 mL each) were collected 5 mins before
404 and within 30 mins after FUS sonication. Blood samples were stored in BD Vacutainer® EDTA
405 (BD Biosciences, San Jose, California) tubes or Cell-Free DNA BCT (Streck Laboratories, La
406 Vista, Nebraska) tubes. Within 4 hrs of collection, whole blood samples were centrifuged at
407 1200xg for 10 mins at 4°C. Isolated plasma was centrifuged a second time at 1800xg for 5 mins
408 at 4°C to further remove cell debris. Plasma aliquots were put on dry ice immediately for snap
409 freezing and stored at -80°C subsequently for later downstream analysis. The plasma-depleted
410 whole blood cells were stored as well for tissue sequencing analysis. After blood collection,
411 craniotomy was performed and the tumor was resected under the guidance of the
412 neuronavigation system. Sonicated and nonsonicated part of tumor tissue was collected from the
413 resected tumor. Skin tissue on the trajectory of FUS sonication was also collected during surgery.

414 The collected tissues were fixed in formalin for paraffin embedding or put in fresh medium for
415 snap freezing.

416 **Plasma protein detection**

417 Frozen plasma samples were thawed at room temperature. All plasma GFAP protein
418 measurements were performed in 2–4 replicates using Simoa® Neurology 2-Plex B Kit on a fully
419 automated HD-X Analyzer (Quanterix, Lexington, MA, United States).

420 **Cell-free DNA extraction and quantification**

421 Plasma/Serum cfc-DNA/cfc-RNA Advanced Fractionation Kit (Norgen Biotek, Thorold, ON,
422 Canada) was used to extract cfDNA from patient plasma per the manufacturer's protocol. cfDNA
423 was eluted in 50 µL of each corresponding buffer and was quantified using Qubit Fluorometric
424 Quantitation (Thermo Fisher Scientific). The 2100 Bioanalyzer (Agilent Technologies) was used
425 to assess the size distribution and concentration of cfDNA extracted from plasma samples. The
426 cfDNA in the mononucleosomal size range (120–180 bp) was determined with the software as
427 the area under the peaks⁵³.

428 **Personalized tumor-informed ctDNA assay**

429 Invitae Personalized Cancer Monitoring (PCM) was adopted to detect the patient-specific tumor
430 variants in patients' plasmas in the following three main steps. First, the data from whole exome
431 sequencing (WES) on tumor and normal (peripheral blood, PB) samples were processed using
432 Invitae's WES Pipeline. The variants identified from the tumor and normal samples were then
433 compared to identify patient-specific tumor variants. Variant calls were used as input for the
434 minimal residual disease (MRD) Panel Designer pipeline. Second, patient-specific panels (PSPs)
435 were designed to target up to 50 patient-specific single tumor variants. The Panel Designer
436 identified high-confidence patient-specific tumor variants which could be targeted using an
437 Anchored Multiplex PCR (AMP) panel. Third, cfDNAs were extracted from the patient's plasma

438 samples and used as input for an AMP library preparation using the personalized panel designed
439 for the patient. Libraries were sequenced using the NovaSeq 6000 sequencing platform (Illumina,
440 San Diego, CA, USA) and the resulting fastq files were analyzed using the Invitae MRD calling
441 pipeline. The MRD analysis pipeline aligns MRD library sequences to the genome, calculates the
442 error rates for the targeted variants and measures the allele fraction for the targeted variants. The
443 observed allele fractions are compared to the background error rate to determine the MRD call.
444 To calculate the concentrations of patient-specific tumor variants in each plasma, the value of
445 alternative observations (AOs) from the Invitae MRD was normalized to the input volume of
446 plasma (tumor variant copies/ml plasma).

447 **ddPCR assays**

448 Custom sequence-specific primers and fluorescent probes were designed and synthesized for
449 patient-specific variant detection (Sigma Aldrich). The forward and reverse primer and probe
450 sequences are listed in **Table S2**. ddPCR reactions were prepared with 2× ddPCR Supermix for
451 probes (no dUTP) (Bio-Rad, Hercules, CA, USA), 2 µL of target cfDNA product, 0.1 µM forward
452 and reverse primers, and 0.1 µM probes. Alternatively, 100 µM 7-deaza-dGTP (New England
453 Biolabs, Beverly, MA, USA) was added to improve PCR amplification for GC rich regions. The
454 QX200 manual droplet generator (Bio-Rad, Hercules, CA, USA) was used to generate droplets.
455 The PCR step was performed on a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA)
456 by use of the following program: 1 cycle at 95°C for 10 mins, 48 cycles at 95°C for 30 s and 60°C
457 for 1 min, 1 cycle at 98°C for 10 mins, and 1 cycle at 4°C infinite, all at a ramp rate of 2°C/s. All
458 plasma samples were analyzed in technical duplicate or triplicate based on sample availability.
459 Data were acquired on the QX200 droplet reader (Bio-Rad, Hercules, CA, USA) and analyzed
460 using QuantaSoft Analysis Pro (Bio-Rad, Hercules, CA, USA). All results were manually reviewed
461 for false positives and background noise droplets based on negative and positive control samples.
462 Tumor variant ctDNA concentrations (copies/ml plasma) were calculated by multiplying the

463 concentration (provided by QuantaSoft) by elution volume, divided by the input plasma volume
464 used during cfDNA extraction.

465 **Bulk RNA sequencing and analysis**

466 Bulk RNA sequencing and analysis were conducted at Genome Technology Access Center at
467 the McDonnell Genome Institute (GTAC@MGI) at Washington University in St. Louis. According
468 to the manufacturer's instructions, snap-frozen sonicated and nonsonicated tumor tissues were
469 homogenized and isolated using the RNeasy MiniPlus Kit (Qiagen, Hilden, Germany). Total RNA
470 integrity was determined using Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).
471 Ribosomal RNA was removed by a hybridization method using Ribo-ZERO kits (Illumina-
472 EpiCentre, San Diego, CA, USA). mRNA was reverse transcribed to yield cDNA using
473 SuperScript III RT enzyme (Thermo Fisher Scientific, Carlsbad, CA, USA) and random hexamers.
474 A second strand reaction was performed to yield double-stranded cDNA. cDNA was blunt-ended,
475 had an A base added to the 3' ends, and then had Illumina sequencing adapters ligated to the
476 ends. Ligated fragments were then amplified for 12-15 cycles using primers incorporating unique
477 dual index tags. Fragments were sequenced on an Illumina NovaSeq-6000 (Illumina, San Diego,
478 CA, USA) using paired-end reads extending 150 bases. Basecalls and demultiplexing were
479 performed with Illumina's bcl2fastq2 software. RNA-seq reads were then aligned and quantitated
480 to the Ensembl release 101 primary assembly with an Illumina DRAGEN Bio-IT on-premise server
481 running version 3.9.3-8 software.

482 All gene counts were then imported into the R/Bioconductor package EdgeR and TMM
483 normalization size factors were calculated to adjust for samples for differences in library size.
484 Ribosomal genes and genes not expressed in the smallest group size minus one sample greater
485 than one count per million were excluded from further analysis. The TMM size factors and the
486 matrix of counts were then imported into the R/Bioconductor package Limma. Weighted
487 likelihoods based on the observed mean-variance relationship of every gene and sample were

488 then calculated for all samples and the count matrix was transformed to moderated log 2 counts-
489 per-million with Limma's voomWithQualityWeights. The performance of all genes was assessed
490 with plots of the residual standard deviation of every gene to their average log count with a
491 robustly fitted trend line of the residuals. Differential expression analysis was then performed to
492 analyze for differences between conditions and the results were filtered for only those genes with
493 Benjamini-Hochberg false-discovery rate adjusted P values less than or equal to 0.05.

494 To identify differentially expressed genes (DEGs), we applied strict criteria: \log_2 (fold-change) >
495 2 and $p < 0.05$ for upregulated genes, and \log_2 (fold-change) < -2 and $p < 0.05$ for downregulated
496 genes. Gene ontology (GO) enrichment analysis was performed using the g:Profiler tool
497 (<https://biit.cs.ut.ee/gprofiler/>) on the DEG list classified into upregulated and downregulated lists.
498 The results were visualized as bar plots or dot plots, which were generated in R using ggplot.
499 Raw data were evaluated for statistical significance with a threshold of $p < 0.05$, using an
500 independent *t*-test to compare fold changes.

501 **Histological analysis**

502 Brain tumor tissue from sonicated and nonsonicated regions were resected and fixed in formalin
503 for paraffin embedding. The brain tumor tissue samples were sectioned to 10 μm slices for
504 hematoxylin and eosin (H&E) staining to examine red blood cell extravasation and cellular injury.
505 Digital images of tissue sections were obtained using an all-in-one microscope (BZ-X810,
506 Keyence, Osaka, Japan). Histological images were assessed by an experienced clinical
507 neuropathologist.

508 **Statistical analysis**

509 Statistics analysis was performed in Graphpad (Prism) (Graphpad, Boston, MA, USA). Unpaired
510 parametric *t*-test were used to compare the level of GFAP and cfDNA in post-FUS plasmas with
511 that in pre-FUS plasma. Paired parametric *t*-test were conducted to compare the concentrations

512 of tumor variants in post-FUS plasmas with that in pre-FUS plasma. All reported P values are two-
513 tailed unless otherwise specified.

514

515 **References**

- 516 1. Jelski, W. & Mroczko, B. Molecular and circulating biomarkers of brain tumors. *Int. J. Mol.*
517 *Sci.* **22**, 1–11 (2021).
- 518 2. Chaudhuri, A. A. *et al.* Early detection of molecular residual disease in localized lung
519 cancer by circulating tumor DNA profiling. *Cancer Discov.* **7**, 1394–1403 (2017).
- 520 3. Bunda, S. *et al.* Liquid biomarkers for improved diagnosis and classification of CNS
521 tumors. *Int. J. Mol. Sci.* **22**, 4548 (2021).
- 522 4. Kwapisz, D. The first liquid biopsy test approved. Is it a new era of mutation testing for
523 non-small cell lung cancer? *Ann. Transl. Med.* **5**, 46 (2017).
- 524 5. Alix-Panabières, C. & Pantel, K. Clinical applications of circulating tumor cells and
525 circulating tumor DNA as liquid biopsy. *Cancer Discov.* **6**, 479–491 (2016).
- 526 6. Bettegowda, C. *et al.* Detection of circulating tumor DNA in early- and late-stage human
527 malignancies. *Sci Transl Med* **6**, 224ra24 (2014).
- 528 7. Chen, W. W. *et al.* Beaming and droplet digital PCR analysis of mutant IDH1 mRNA in
529 glioma patient serum and cerebrospinal fluid extracellular vesicles. *Mol. Ther. - Nucleic*
530 *Acids* **2**, e109 (2013).
- 531 8. Boisselier, B. *et al.* Detection of IDH1 mutation in the plasma of patients with glioma.
532 *Neurology* **79**, 1693–1698 (2012).
- 533 9. Saenz-Antoñanzas, A. *et al.* Liquid biopsy in glioblastoma: Opportunities, applications
534 and challenges. *Cancers (Basel)*. **11**, 950 (2019).
- 535 10. Bettegowda, C. *et al.* Detection of circulating tumor DNA in early- and late-stage human
536 malignancies. *Sci. Transl. Med.* **6**, (2014).
- 537 11. Connolly, I. D., Li, Y., Gephart, M. H. & Nagpal, S. The “liquid biopsy”: The role of
538 circulating DNA and RNA in central nervous system tumors. *Curr. Neurol. Neurosci. Rep.*
539 **16**, 1–8 (2016).
- 540 12. Gorick, C. M. *et al.* Applications of focused ultrasound-mediated blood-brain barrier
541 opening. *Adv. Drug Deliv. Rev.* **191**, 114583 (2022).
- 542 13. Chen, H., Kreider, W., Brayman, A. A., Bailey, M. R. & Matula, T. J. Blood vessel
543 deformations on microsecond time scales by ultrasonic cavitation. *Phys. Rev. Lett.* **106**,
544 034301 (2011).
- 545 14. Hynynen, K., McDannold, N., Vykhodtseva, N. & Jolesz, F. A. Noninvasive MR imaging-
546 guided focal opening of the blood-brain barrier in rabbits. *Radiology* **220**, 640–646
547 (2001).
- 548 15. Mainprize, T. *et al.* Blood-brain barrier opening in primary brain tumors with non-invasive
549 MR-guided focused ultrasound: A clinical safety and feasibility study. *Sci. Rep.* **9**, 321
550 (2019).
- 551 16. Park, S. H. *et al.* One-year outcome of multiple blood–brain barrier disruptions with
552 temozolomide for the treatment of glioblastoma. *Front. Oncol.* **10**, 1–7 (2020).
- 553 17. Park, S. H. *et al.* Safety and feasibility of multiple blood-brain barrier disruptions for the
554 treatment of glioblastoma in patients undergoing standard adjuvant chemotherapy. *J.*

- 555 *Neurosurg.* **134**, 475–483 (2021).
- 556 18. Chen, K. T. *et al.* Neuronavigation-guided focused ultrasound for transcranial blood-brain
557 barrier opening and immunostimulation in brain tumors. *Sci. Adv.* **7**, eabd0772 (2021).
- 558 19. Meng, Y. *et al.* MR-guided focused ultrasound enhances delivery of trastuzumab to Her2-
559 positive brain metastases. *Sci. Transl. Med.* **13**, 1–9 (2021).
- 560 20. Anastasiadis, P. *et al.* Localized blood-brain barrier opening in infiltrating gliomas with
561 MRI-guided acoustic emissions-controlled focused ultrasound. *Proc. Natl. Acad. Sci. U.*
562 *S. A.* **118**, (2021).
- 563 21. Lipsman, N. *et al.* Blood–brain barrier opening in Alzheimer’s disease using MR-guided
564 focused ultrasound. *Nat. Commun.* **9**, 2336 (2018).
- 565 22. Abrahao, A. *et al.* First-in-human trial of blood-brain barrier opening in amyotrophic lateral
566 sclerosis using MR-guided focused ultrasound. *Nat. Commun.* **10**, 4373 (2019).
- 567 23. Gasca-Salas, C. *et al.* Blood-brain barrier opening with focused ultrasound in Parkinson’s
568 disease dementia. *Nat. Commun.* **12**, 1–7 (2021).
- 569 24. Zhu, L. *et al.* Focused ultrasound-enabled brain tumor liquid biopsy. *Sci. Rep.* **8**, 6553
570 (2018).
- 571 25. Pan, S. *et al.* Leptomeningeal disease and tumor dissemination in a murine diffuse
572 intrinsic pontine glioma model: implications for the study of the tumor-cerebrospinal fluid-
573 ependymal microenvironment. *Neuro-Oncology Adv.* **4**, 1–12 (2022).
- 574 26. Zhu, L. *et al.* Focused ultrasound for safe and effective release of brain tumor biomarkers
575 into the peripheral circulation. *PLoS One* **15**, e0234182 (2020).
- 576 27. Pacia, C. P. *et al.* Feasibility and safety of focused ultrasound-enabled liquid biopsy in the
577 brain of a porcine model. *Sci. Rep.* **10**, 7449 (2020).
- 578 28. Pacia, C. P. *et al.* Focused Ultrasound-mediated Liquid Biopsy in a Tauopathy Mouse
579 Model. *Radiology* 220869 (2023) doi:10.1148/radiol.220869.
- 580 29. Pacia, C. P. *et al.* Sonobiopsy for minimally invasive, spatiotemporally-controlled, and
581 sensitive detection of glioblastoma-derived circulating tumor DNA. *Theranostics* **12**, 362–
582 378 (2022).
- 583 30. Meng, Y. *et al.* MR-guided focused ultrasound liquid biopsy enriches circulating
584 biomarkers in patients with brain tumors. *Neuro. Oncol.* **23**, 1789–1797 (2021).
- 585 31. Jameel, A., Bain, P., Nandi, D., Jones, B. & Gedroyc, W. Device profile of exAblate Neuro
586 4000, the leading system for brain magnetic resonance guided focused ultrasound
587 technology: an overview of its safety and efficacy in the treatment of medically refractory
588 essential tremor. *Expert Rev. Med. Devices* **18**, 429–437 (2021).
- 589 32. Abdelhak, A. *et al.* Blood GFAP as an emerging biomarker in brain and spinal cord
590 disorders. *Nat. Rev. Neurol.* 2022 183 **18**, 158–172 (2022).
- 591 33. Muralidharan, K. *et al.* TERT promoter mutation analysis for blood-based diagnosis and
592 monitoring of gliomas. *Clin. Cancer Res.* **27**, 169–178 (2021).
- 593 34. Gao, K. *et al.* TERT promoter mutations and long telomere length predict poor survival
594 and radiotherapy resistance in gliomas. *Oncotarget* **7**, 8712–8725 (2016).

- 595 35. Reuss, D. E. Updates on the WHO diagnosis of IDH-mutant glioma. *J. Neurooncol.*
596 (2023) doi:10.1007/s11060-023-04250-5.
- 597 36. McMahon, D. & Hynynen, K. Acute inflammatory response following increased blood-
598 brain barrier permeability induced by focused ultrasound is dependent on microbubble
599 dose. *Theranostics* **7**, 3989–4000 (2017).
- 600 37. Kovacs, Z. I. *et al.* Disrupting the blood-brain barrier by focused ultrasound induces
601 sterile inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E75–E84 (2017).
- 602 38. Choi, H. J. *et al.* The new insight into the inflammatory response following focused
603 ultrasound-mediated blood–brain barrier disruption. *Fluids Barriers CNS* **19**, 1–21 (2022).
- 604 39. Chien, C. Y., Xu, L., Pacia, C. P., Yue, Y. & Chen, H. Blood–brain barrier opening in a
605 large animal model using closed-loop microbubble cavitation-based feedback control of
606 focused ultrasound sonication. *Sci. Rep.* **12**, 1–9 (2022).
- 607 40. Chien, C.-Y., Yang, Y., Gong, Y., Yue, Y. & Chen, H. Blood-Brain Barrier Opening by
608 Individualized Closed-Loop Feedback Control of Focused Ultrasound. *BME Front.* **2022**,
609 1–11 (2022).
- 610 41. Larribère, L. & Martens, U. M. Advantages and challenges of using ctDNA NGS to assess
611 the presence of minimal residual disease (MRD) in solid tumors. *Cancers (Basel)*. **13**,
612 (2021).
- 613 42. Newman, A. M. *et al.* Integrated digital error suppression for improved detection of
614 circulating tumor DNA. *Nat. Biotechnol.* **34**, 547–555 (2016).
- 615 43. Reinert, T. *et al.* Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients
616 with Stages I to III Colorectal Cancer. *JAMA Oncol.* **5**, 1124–1131 (2019).
- 617 44. Keller, L., Belloum, Y., Wikman, H. & Pantel, K. Clinical relevance of blood-based ctDNA
618 analysis: mutation detection and beyond. *Br. J. Cancer* **124**, 345–358 (2021).
- 619 45. Wu, J. *et al.* Tumor circulome in the liquid biopsies for cancer diagnosis and prognosis.
620 *Theranostics* **10**, 4544–4556 (2020).
- 621 46. Rolfo, C. *et al.* Liquid Biopsy for Advanced Non-Small Cell Lung Cancer (NSCLC): A
622 Statement Paper from the IASLC. *J. Thorac. Oncol.* **13**, 1248–1268 (2018).
- 623 47. Nagel, S. *et al.* Minocycline and hypothermia for reperfusion injury after focal cerebral
624 ischemia in the rat—Effects on BBB breakdown and MMP expression in the acute and
625 subacute phase. *Brain Res.* **1188**, 198–206 (2008).
- 626 48. Zhang, Y. *et al.* Molecular Identity Changes of Tumor-Associated Macrophages and
627 Microglia After Magnetic Resonance Imaging–Guided Focused Ultrasound–Induced
628 Blood–Brain Barrier Opening in a Mouse Glioblastoma Model. *Ultrasound Med. Biol.*
629 (2023) doi:10.1016/J.ULTRASMEDBIO.2022.12.006.
- 630 49. Xu, L. *et al.* Characterization of the Targeting Accuracy of a Neuronavigation-guided
631 Transcranial FUS system in vitro, in vivo, and in silico. *IEEE Trans. Biomed. Eng.*
632 (2022) doi:10.1109/TBME.2022.3221887.
- 633 50. Chen, K.-T. *et al.* Neuronavigation-guided focused ultrasound for transcranial blood-brain
634 barrier opening and immunostimulation in brain tumors. *Sci. Adv.* **7**, eabd0772 (2021).
- 635 51. Poulipoulos, A. N. *et al.* Safety evaluation of a clinical focused ultrasound system for

- 636 neuronavigation guided blood-brain barrier opening in non-human primates. *Sci. Rep.* **11**,
637 1–17 (2021).
- 638 52. Yang, Y. *et al.* Static magnetic fields dampen focused ultrasound- mediated blood-brain
639 barrier opening. *Radiology* **300**, 681 (2021).
- 640 53. Mouliere, F. *et al.* Fragmentation patterns and personalized sequencing of cell-free DNA
641 in urine and plasma of glioma patients. *EMBO Mol. Med.* **13**, e12881 (2021).
- 642
- 643

644 **Acknowledgments**

645 This work was supported by the National Institutes of Health R01CA276174, R01MH116981,
646 UG3MH126861, R01EB027223, and R01EB030102.

647 **Author contributions**

648 Conceptualization: ECL, HC Experiment design: ECL, HC Experiment implementation: ECL, HC,
649 UA, XL, CYC, JY, SF, AS. Result Investigation: HC, XL, CYC, JY, KS. Funding acquisition: ECL,
650 HC. Project administration: ECL, HC. Supervision: ECL, HC. Writing – original draft: HC, JY, XL,
651 CYC Writing – review & editing: ECL, HC, JY, XL, CYC, YY, SF, AC, KS, AN, RD, UA.

652 **Competing interests**

653 ECL and HC have a patent on the sonobiopsy technique (US20190323086A1).

654 **Materials & Correspondence.**

655 All data that support the findings of this study are available from the corresponding authors upon
656 reasonable request.

657 **Table 1. Patient demographics**

Patient	Sex	Age range	Diagnosis	IDH-1	TERT
G01	M	71–75	Glioblastoma	Wild type	TERT C228T
G02	M	56–60	Glioblastoma	Wild type	TERT C250T
G03	F	66–70	Glioblastoma	Wild type	TERT C228T

658

659

660

661 **Table 2. Summary of sonobiopsy parameters**

Patient number	G01	G02	G03	662
Simulated in situ pressure (MPa)	0.49	0.31	0.66	663
Estimated mechanical index (MI)	0.61	0.38	0.82	
Procedure duration from the time when the patient was ready to the end of FUS sonication (min)	24	14	30	664 665
Microbubble dose ($\mu\text{L}/\text{Kg}$)	10	10	10	666
Microbubble volume (mL)	5.3	8.2	4.54	
Stable cavitation dose (a.u.)	4.2	1.9	6.9	667
Inertial cavitation dose (a.u.)	0.41	0.39	79.9	668

669

670

671

672

673 **Figure captions**

674 **Figure 1. Sonobiopsy procedure. (a)** Contrast-enhanced T1w MRI images prior to sonobiopsy
675 for three patients (G01, G02, and G03) enrolled in this study. **(b)** Illustration of the
676 neuronavigation-guided sonobiopsy setup. **(c)** Sonobiopsy clinical workflow.

677 **Figure 2. Tumor targeting and treatment monitoring (a)** Screenshot from the Stealth
678 neuronavigation system to show the precise alignment of the FUS focus (arrow) with the planned
679 target indicated by the crossing point of the red lines. **(b)** 3D reconstruction to show the spatial
680 location of the planned target inside the tumor and the simulated FUS focus location based on
681 the trajectory obtained from the neuronavigation system. **(c)** Representative time-frequency
682 analysis of the acquired cavitation signal during FUS sonication to show the spectrum of the
683 signals. **(d)** Representative stable cavitation and inertial cavitation levels measured based on the
684 frequency spectrum of the acquired cavitation signals.

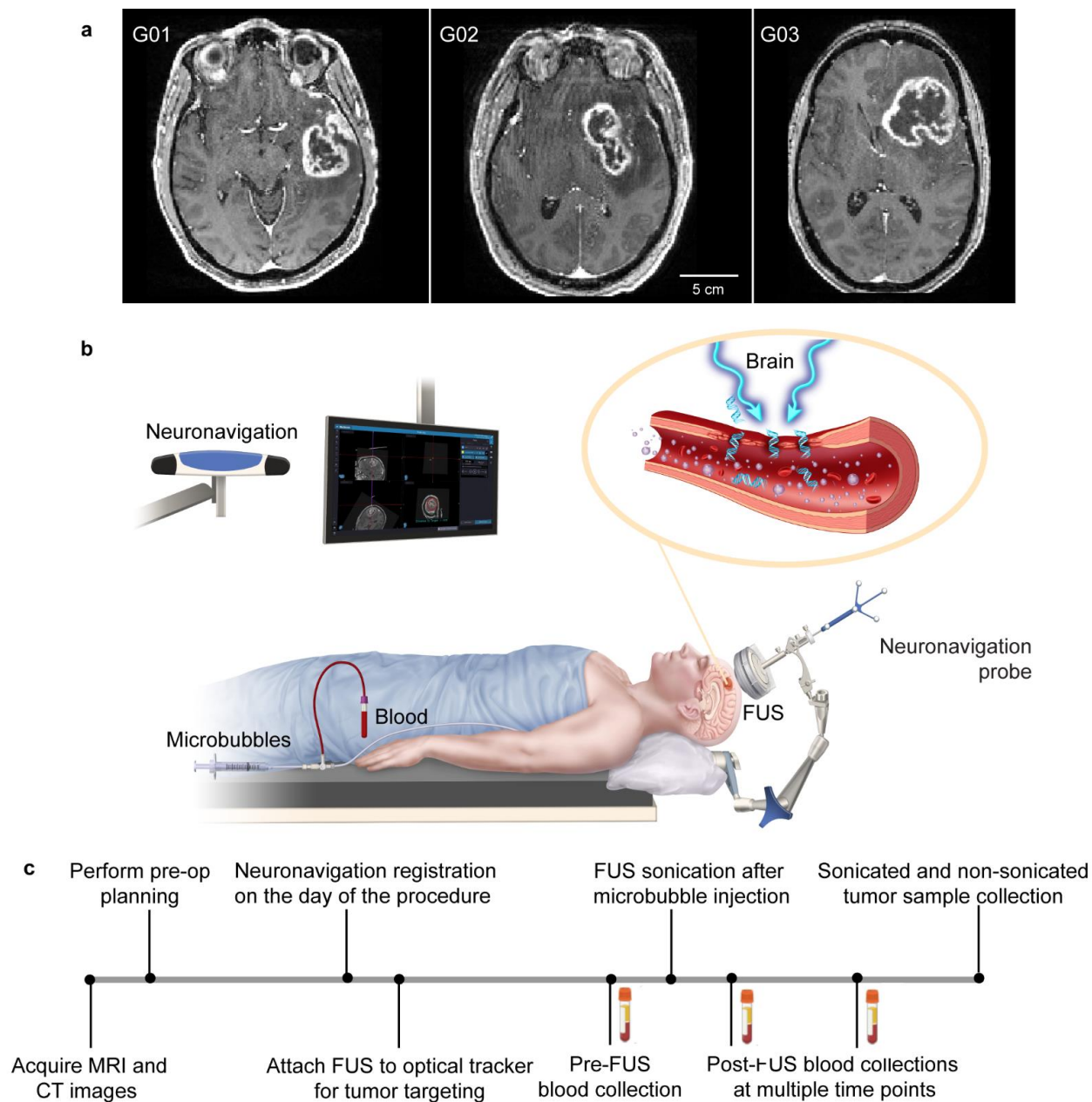
685 **Figure 3. Sonobiopsy enriched circulating GBM-specific biomarkers. (a)** Plasma GFAP
686 concentration measured at 5 mins pre-FUS (-5 min) and different time points post-FUS (5, 10,
687 and/or 30 mins) for three patients (G01, G02, and G03) [unpaired parametric *t*-test comparing
688 post-FUS each time point with pre-FUS, * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ***** $p < 0.0001$, bar
689 graph represents mean \pm standard deviation (SD)]. **(b)** Concentration of single nucleosome length
690 cfDNA (120–280 bp fragments) was significantly increased after FUS for all three patients
691 (unpaired parametric *t*-test comparing post-FUS time points with pre-FUS). **(c)** Patient-specific
692 tumor variants detected using a personalized tumor-informed ctDNA assay were significantly
693 increased for G02 and G03 (paired parametric *t*-test comparing post-FUS time points with pre-
694 FUS). **(d)** ddPCR analysis of plasma TERT and IDH1 shows sonobiopsy increased the level of
695 TERT mutation, but not the wild type IDH1.

696 **Figure 4. Safety of sonobiopsy by tissue analysis. (a)** Gross pathology examination of the
697 surface of the brain after craniotomy for the three patients (G01, G02, and G03) did not observe
698 tissue damage induced by the FUS procedure. **(b)** Hematoxylin and eosin (H&E) staining of the
699 sonication and nonsonicated brain tumor tissue did not observe clear evidence of tissue damage.

700 **Figure 5. Transcriptome analysis of differentially expressed genes (DEGs) after**
701 **sonobiopsy. (a)** Hierarchical clustering heat map of all DEGs with log₂FC (fold change) values >
702 2 and p < 0.05. Rows represent brain tissue samples acquired from the FUS-sonicated (FUS) or
703 nonsonicated (CON) tumor regions from different patients, and columns represent individual
704 DEGs. **(b)** Summary of the total number of upregulated and downregulated DEGs. **(c)**
705 Upregulated (red) and downregulated (blue) DEGs with GO functional analysis for enriched
706 biological processes, cellular components, and molecular functions. *p < 0.05, **p < 0.01,
707 ***p < 0.001, ****p < 0.0001. **(d)** Upregulated and downregulated genes involved in GO terms
708 related to cell physical structure. **(e)** Upregulated and downregulated genes involved in GO terms
709 related to inflammatory or immune-related response.

710

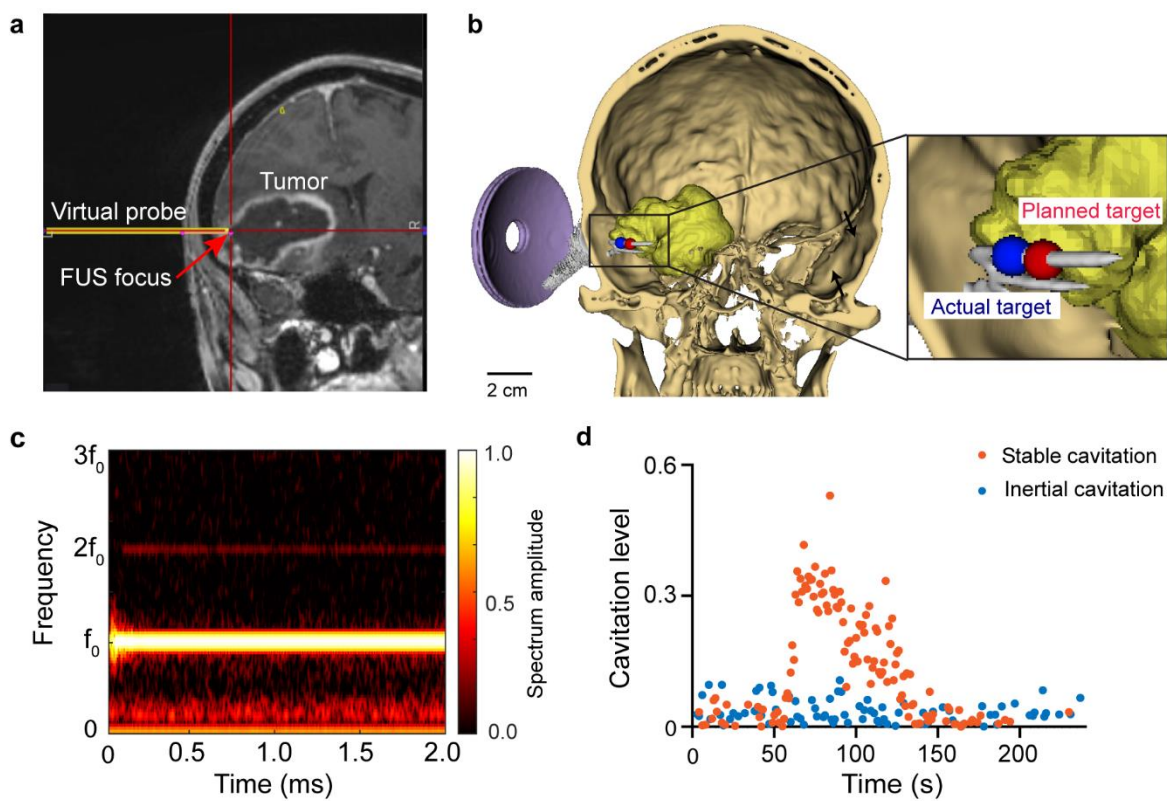
711 **Figure 1**



712

713

714 **Figure 2**

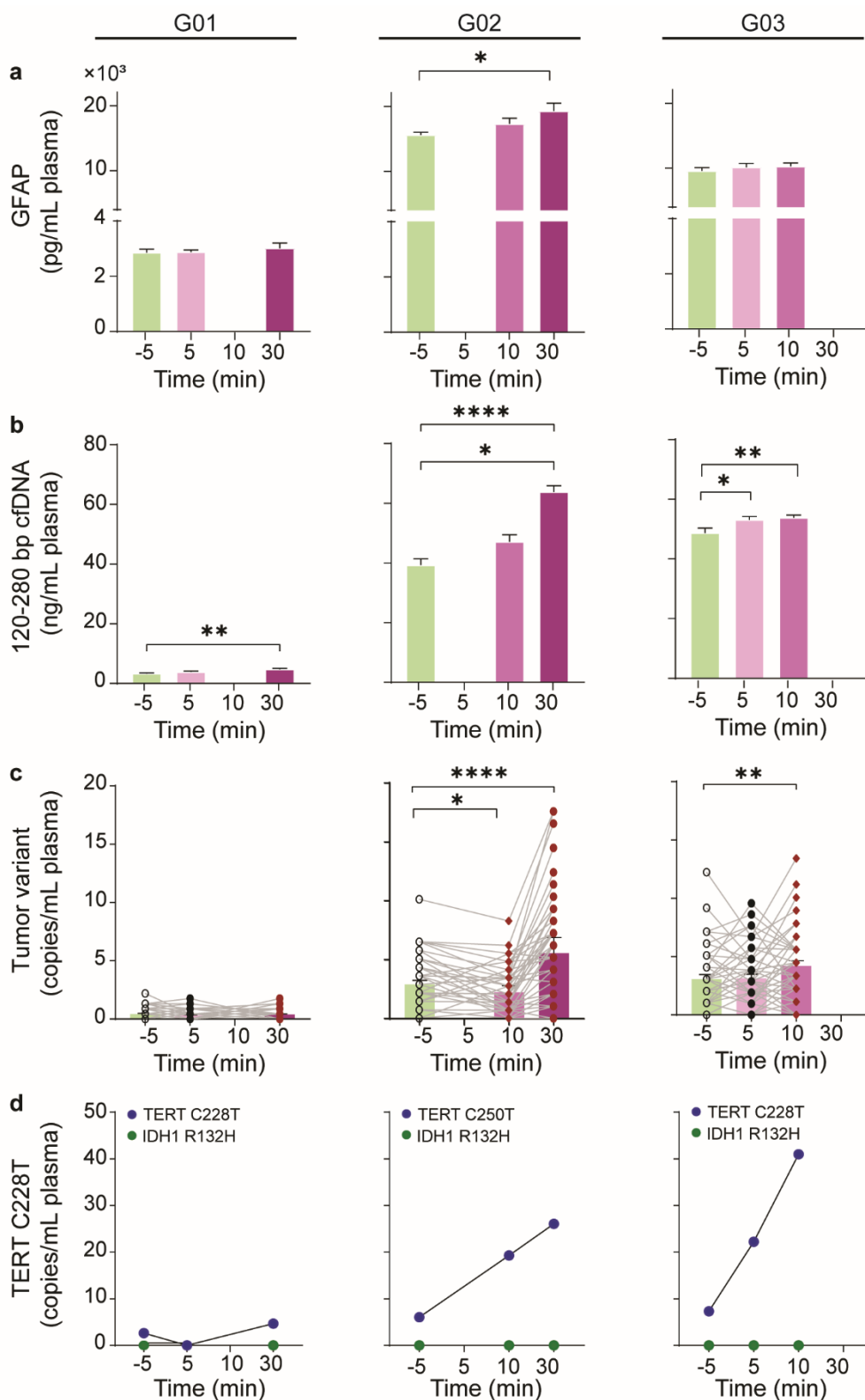


715

716

717

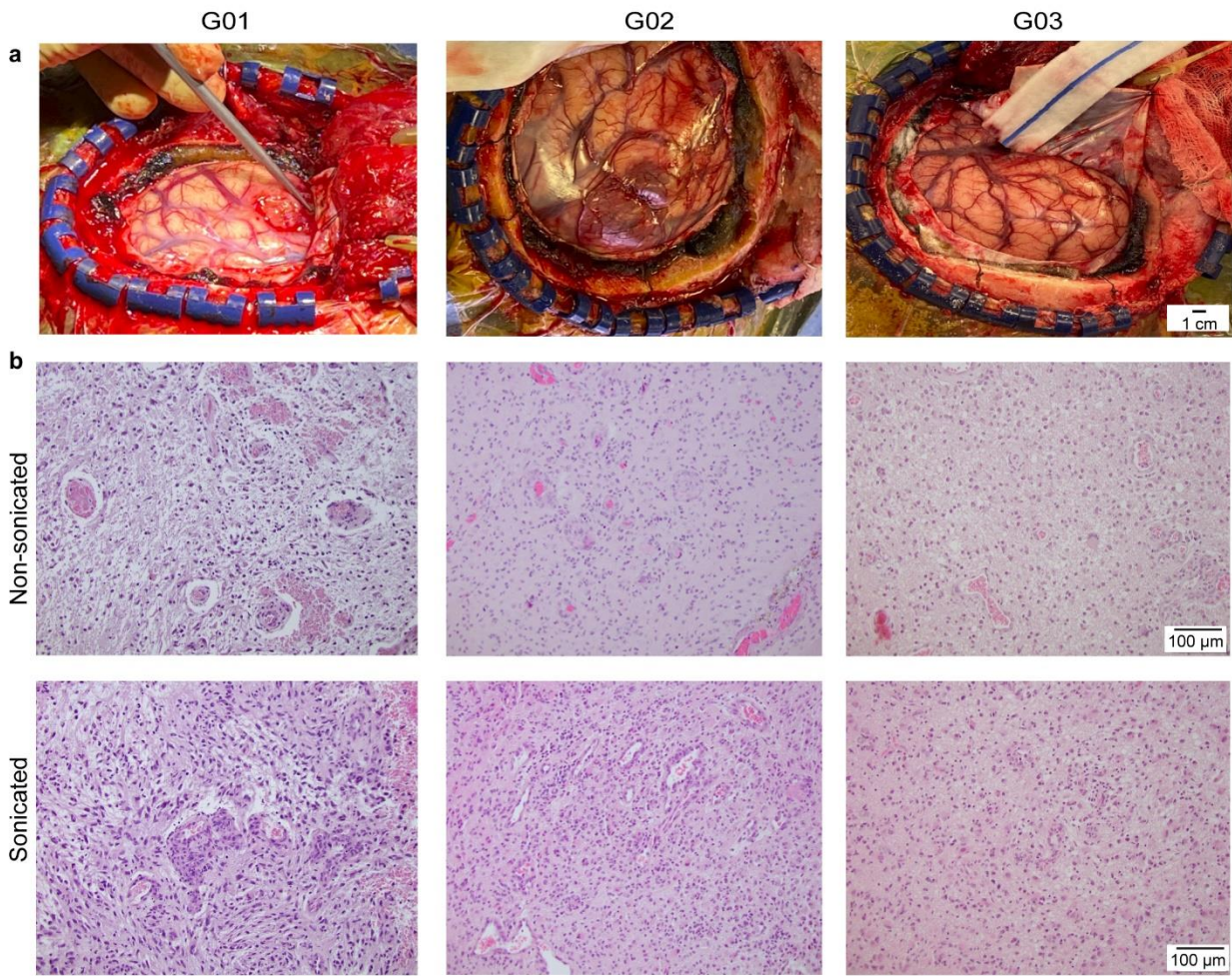
718 **Figure 3**



719

720

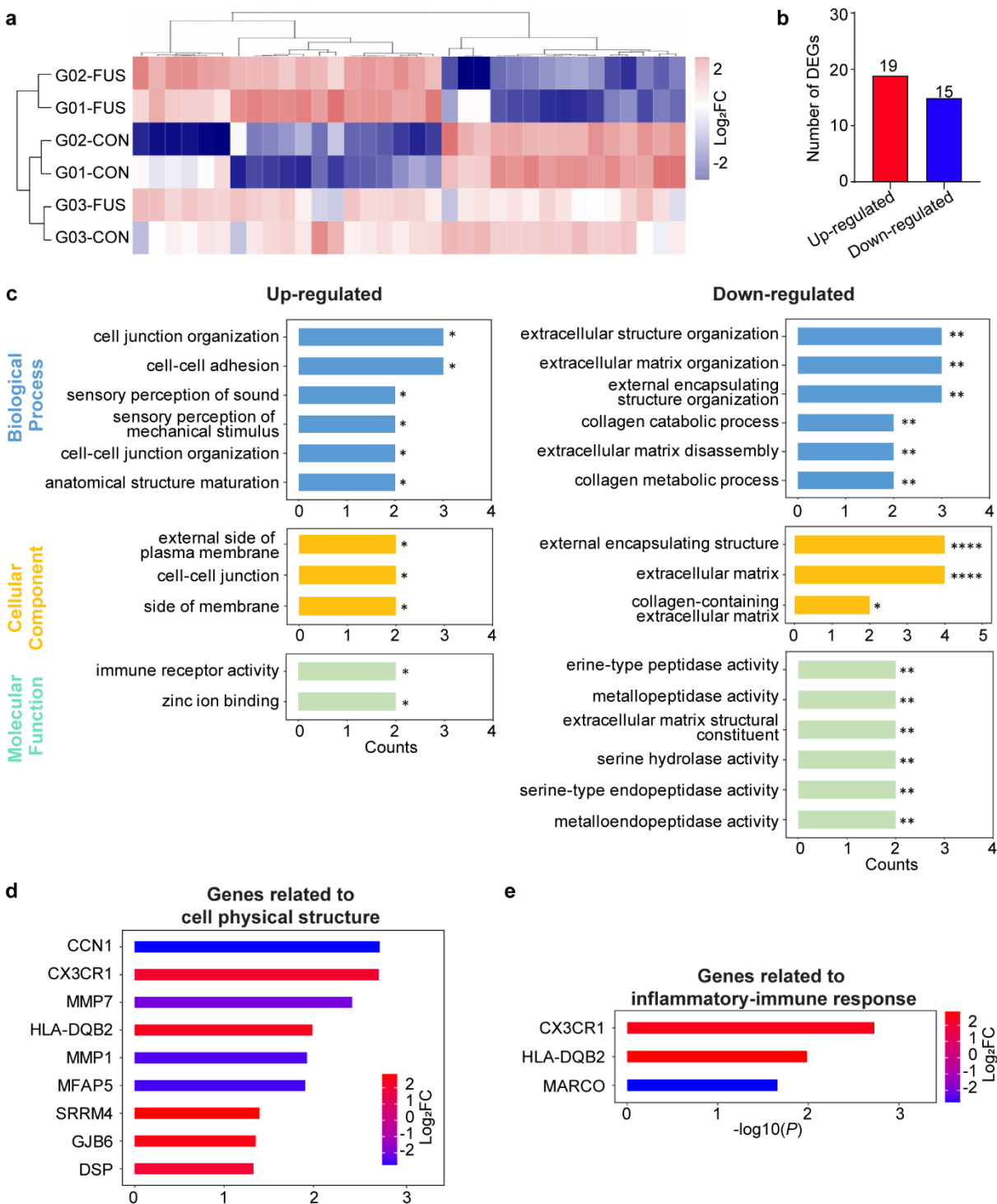
721 **Figure 4**



722

723

724 **Figure 5**



725

726

727