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1 Impact of blood-brain barrier permeabilization induced by ultrasound associated to
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4

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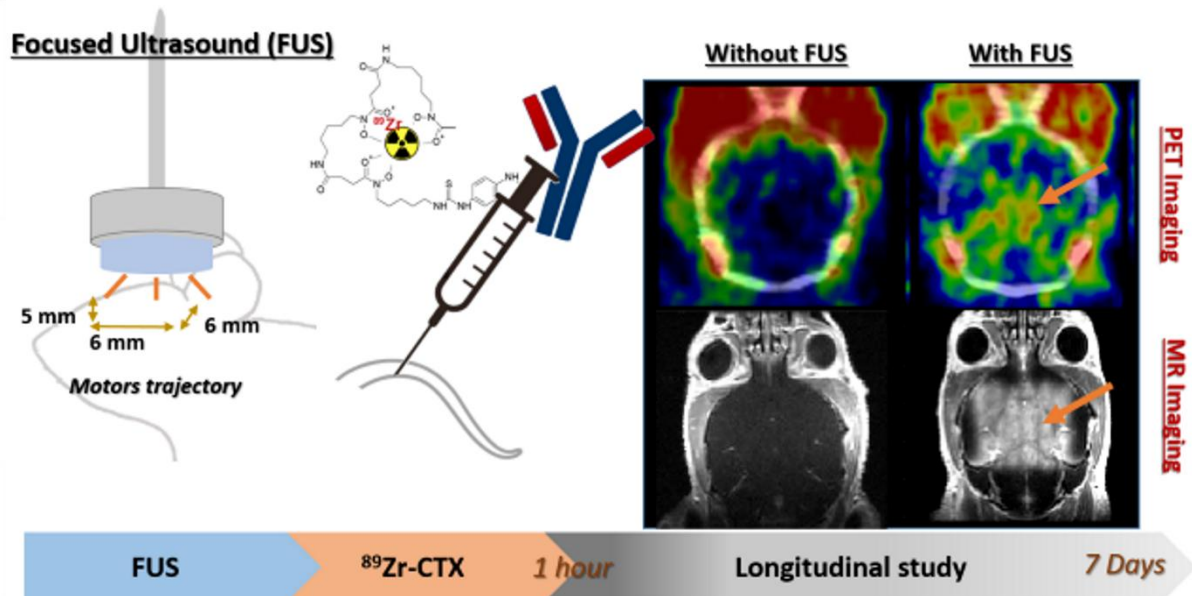
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18 **Abstract:**



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22 **Abstract**

23 Epidermal growth factor receptor (EGFR), involved in cell proliferation and migration, is
24 overexpressed in ~50% of glioblastomas. Anti-EGFR based strategies using monoclonal antibodies
25 (mAb) such as cetuximab (CTX) have been proposed for central nervous system (CNS) cancer
26 therapy. However, the blood-brain barrier (BBB) drastically restricts their brain penetration which
27 limits their efficacy for the treatment of glioblastomas. Herein, a longitudinal PET imaging study was
28 performed to assess the relevance and the impact of focused ultrasound (FUS)-mediated BBB
29 permeabilization on the brain exposure to the anti-EGFR mAb CTX over time. For this purpose, FUS
30 permeabilization process with microbubbles was applied on intact BBB mouse brain before the
31 injection of ⁸⁹Zr-labeled CTX for longitudinal imaging monitoring. FUS induced a dramatic increase
32 in mAb penetration to the brain, 2 times higher compared to the intact BBB. The transfer of ⁸⁹Zr-CTX
33 from blood to the brain was rendered significant by FUS ($k_{\text{uptake}} = 1.3 \pm 0.23 \text{ min}^{-1}$ with FUS versus
34 $k_{\text{uptake}} = 0 \pm 0.006 \text{ min}^{-1}$ without FUS). FUS allowed significant and prolonged exposure to mAb in the
35 brain parenchyma. This study confirms the potential of FUS as a target delivery method for mAb in
36 CNS.

37 **Key words:** Antibody, pharmacokinetics, brain delivery, FUS, longitudinal PET imaging, Cetuximab,
38 mAb diffusion.

39

40 **Highlights:**

- 41 ○ FUS and MB significantly increase the delivery of CTX across the BBB.
- 42 ○ PET is a powerful method to assess the PK of the passage of mAb through the BBB.
- 43 ○ ⁸⁹Zr-DFO-CTX was used to follow quantitatively enhanced brain exposure to CTX.
- 44 ○ ImmunoPET-combined FUS is a clinically relevant strategy for GBM innovative therapy.

45

46 I. INTRODUCTION:

47 Epidermal growth factor receptor (EGFR) plays a key role in essential cellular functions that include
48 proliferation and migration. EGFR signaling mediates oncogenic progression and metastasis in several
49 human malignancies including peripheral and CNS cancers such as glioma [1] [2]. *EGFR vIII*
50 (deletions of exons 2-7) is the most prevalent mutation observed for EGFR. *EGFR vIII* is expressed in
51 ~50% of glioblastomas which amplifies the expression of the wild-type protein [3]. Overexpression of
52 EGFR is associated with poor prognosis in glioma patients [4]. The EGFR pathway is therefore
53 regarded as a promising therapeutic target against CNS malignancies. In peripheral cancer, anti-EGFR
54 monoclonal antibodies (mAb) were shown to inhibit cell proliferation, enhance apoptosis, and reduce
55 angiogenesis, invasiveness and metastasis [5]. Anti-EGFR mAbs such as cetuximab (CTX) are active
56 against EGFR vIII and prevent the binding of endogenous ligand [6]. Although anti-EGFR mAbs
57 show favorable effects in colorectal, head and neck and non-small cell lung cancers, clinical trials
58 using CTX in patients with glioblastoma did not show significant improvement over standard of care
59 regimens [7,8]. The blood-brain barrier (BBB) is the main obstacle limiting the passage of therapeutics
60 into the brain [9]. Insufficient exposure of CNS tumors to CTX across the BBB is widely assumed to
61 explain the limited therapeutic efficacy.

62 For glioma, the abnormal tumor vasculature issued from the overproduction of proangiogenic factors
63 leads to a compromised blood-tumor barrier (BTB) which is assumed to allow the extravasation of
64 small and large molecules [10]. However, in reality, the penetration of drugs into the compromised
65 BTB is very low, leading to inefficient tumor treatment. In early stages of glioblastoma (GBM), tumor
66 own vasculature is not yet very well developed and the BTB is apparently not disrupted. At these
67 stages, BTB resembles the BBB and prevents efficient passage of cancer therapeutics, including small
68 molecules and antibodies [11]. Moreover as glioma progresses, BTB presents some degree of
69 heterogeneity i.e. all glioma have clinically significant regions of tumor with an intact BBB limiting
70 the diffusion of treatment [12,13]. As mAb can be blocked from reaching the areas where the BBB
71 remained intact, there is a clear need for strategies that can deliver immunotherapeutic agents into
72 tumors.

73 The reigning paradigm to overcome this blockage shifted to the establishment of a safe and effective
74 method to improve the brain delivery of mAb. Recent preclinical and clinical studies based on osmotic
75 BBB disruption using intra-arterial mannitol infusion provided encouraging results in terms of
76 improved drug delivery and safety, nevertheless these methods do not allow for controlled and
77 localized BBB permeabilization [14]. The combination of injected microbubbles with low intensity
78 focused ultrasound (FUS) is the most advanced method for controlled, minimally-invasive and
79 transient BBB disruption in vivo [15–17]. Low acoustic pressures, produced by FUS, induce
80 microbubble oscillations (alternation of expansion and shrinkage of microbubbles) near the vessel wall
81 that can result in tight junction loosening due to local stress via push-pull mechanisms or
82 microstreaming [18]. In preclinical model, FUS was recently shown to lead to a 3.5 fold increase in
83 the brain concentrations of mAb [19]. However, current methods used to quantify drug concentrations
84 in the brain are invasive or destructive (mass spectrometry, Elisa assay, HPLC). Non-invasive imaging
85 technique such as magnetic resonance imaging (MRI) or positron emission tomography (PET) have
86 therefore been developed to address the brain uptake of labeled mAbs, thus paving the way for the
87 translational study of the neuropharmacokinetics of biologics [20,21]. However, to our knowledge, no
88 *in vivo* imaging was proposed to study brain mAb concentrations over a prolonged time to provide an
89 in-depth understanding of the neuropharmacokinetics and overall brain exposure to mAbs in vivo.

90 In the present study, the immunoPET paradigm was used to evaluate over time (up to 1 week) the
91 impact of FUS-induced BBB opening on the delivery of CTX to the whole brain in mice with intact
92 BBB. We used the long half-life ($t_{1/2} = 78.4$ h) radionuclide zirconium 89 (^{89}Zr) which is relevant to
93 the biological half-life of mAbs. In this work, kinetic modeling of the brain delivery of ^{89}Zr -CTX was
94 performed to describe the initial transfer rate of CTX across the BBB and to address its retention by
95 the brain over time.

96 We have found that FUS can increase significantly the uptake and the transfer rate of labeled mAb.
97 Permeabilized ⁸⁹Zr-DFO-CTX stayed at the proximity of the FUS field for up to 72 h which further
98 consolidate the rationale of using FUS as a controlled delivery method and ⁸⁹Zr PET imaging as a
99 suitable monitoring technique.

100 **II. MATERIALS AND METHODS**

101 **1. Materials**

102 [⁸⁹Zr]Zr-oxalic acid was purchased from PerkinElmer (Netherlands). Erbitux (Cetuximab 5 mg/mL)
103 was purchased from Merck (Belgium). p-isothiocyanatobenzyl-desferrioxamine (p-NCS-Bz-DFO) was
104 purchased from Chematech (Dijon, France). Ectodomain of human and murine EGFR was obtained
105 from Sino Biological Inc. (Beijing, China). Sodium bicarbonate, sodium acetate, gentisic acid,
106 dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). PD-10 desalting
107 columns and iTLC-SG glass microfiber chromatography paper impregnated with silica gel were
108 purchased from GE Healthcare (France). A431 and GL261 cells were obtained from DSMZ
109 (Braunschweig, Germany). Fetal bovine serum (FBS) was purchased from PAA (Coelbe, Germany).
110 L-glutamine, penicillin and streptomycin was obtained from Invitrogen (Frankfurt, Germany). *nu/nu*
111 mice were purchased from Charles River (Écully, France). Microbubbles (SonoVue®) were purchased
112 from Bracco (Milan, Italy). Gadoterate (Dotarem, about 1.66mmol/kg) was obtained from Guerbet
113 (France).

114 **2. CTX affinity for murine and human EGFR.**

115 Binding kinetics of the interaction between CTX and the ectodomain of human and murine EGFR
116 were determined using an Octet RED96 instrument (ForteBio). Anti-hIgG Fc Capture (AHC)
117 Biosensors (ForteBio) were loaded with CRX using a 50 nM solution for 60 sec. After recording a 180
118 s baseline using a kinetic buffer (PBS buffer supplemented with 0.5% (w/v) bovine serum albumin
119 (BSA) and 0.05% (v/v) Tween 20), the association of EGFR was measured at different concentrations
120 for 200 s before a 2000 s dissociation step in kinetic buffer. Five concentrations of human EGFR
121 ectodomain (25nM, 12.5 nM, 6.25nM, 3.125 nM, and 1.6 nM) and four concentrations of murine
122 EGFR ectodomain (400 nM, 200nM, 100 nM and 50 nM) were tested. All measurements were
123 performed at 25°C temperature in 96-well microplates. Data were analyzed using a 1:1 interaction
124 model on the ForteBio data analysis software 10.

125 **3. Radiolabeling of CTX with ⁸⁹Zr.**

126 Cetuximab was radiolabeled with ⁸⁹Zr using p-NCS-Bz- (DFO) as chelate according to a previously
127 described protocol.[22] Briefly, CTX (5 mg/mL) was dispersed in 200 µL of 0.1 M sodium
128 bicarbonate buffer (pH 9.0). The final reaction mixture was adjusted to a total volume of 0.5 mL by
129 adding 0.1 M sodium bicarbonate buffer. Four equivalents of DFO (in DMSO) were added to the
130 antibody solution dropwise while mixing at 500 r.p.m. The reaction mixture was purified by running
131 through a PD-10 column using sodium acetate containing gentisic acid as mobile phase (5 mg.mL⁻¹
132 gentisic acid in 0.25 M sodium acetate (pH 5.4–5.6). Preparation of functionalized CTX (CTX-DFO)
133 was stored at -20°C. On the day of imaging experiments, CTX-DFO was radiolabeled with ⁸⁹Zr (111
134 MBq) at 37°C for 60 min. ⁸⁹Zr-DFO-CTX was purified through PD-10 column using gentisic acid
135 solution. The pH was carefully adjusted using a pH meter equipped with a micro pH electrode during
136 the critical steps of the antibody functionalization and radiolabeling. For the radiolabeling, the pH was
137 adjusted at 7.2 thanks to HEPES solution at 0.5M (Sigma, France) for optimal labeling efficiency. The
138 radiochemical yield and purity were determined using instant thin- layer chromatography (ITLC) with
139 iTLC-SG glass microfiber chromatography paper impregnated with silica gel as stationary phase and
140 20 mM citric acid (pH 4.9–5.1) as mobile phase. ITLC The migration was followed by radio- TLC
141 detection (Mini-Scan TLC Imaging Scanner, Eckert&Ziegler, Berlin, Germany).

142 **4. Cell cultivation**

143 A431 cells (ATCC, CRL1555) and GL261 cells (DMZE, ACC 802) were cultured in DMEM high
144 glucose (4500mg.L⁻¹) supplemented with 10% fetal bovine serum (FBS), GlutaMAX™ (862 mg.L⁻¹),
145 and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 95% air and 5%
146 carbon dioxide (CO₂).

147 **5. Functional Binding Assay of ⁸⁹Zr-DFO-CTX.**

148 The immunoreactivity of the antibody for EGFR receptors after introduction of DFO and ⁸⁹Zr was
149 assessed prior to any *in vivo* experiments. This assay was performed by a standard Lindmo excess
150 antigen method involving the *in vitro* labeled CTX and EGFR overexpressing cells A431 [23]. These
151 cells were established from a human epidermoid carcinoma expressing abnormally high levels of the
152 EGFR. Briefly, 20 ng of radiolabeled CTX were added to 0.5–5x10⁷ A431 cells in 0.5 ml of medium.
153 Cells were incubated for 60 min at room temperature with continuous mixing throughout to keep them
154 in suspension. Then, cells were harvested by centrifugation and washed once to remove unbound
155 antibody, and pellets were measured in a gamma counter (Cobra II, Canberra-Packard, Schwadorf,
156 Austria). The percentage of ⁸⁹Zr-DFO-CTX bound to A431 cells was calculated as the ratio of
157 radioactivity in cell pellet to the mean total radioactivity associated with radiolabeled antibodies. The
158 total applied radioactivity over specific bound radioactivity was plotted against the inverse of cell
159 concentration. Fitting of a straight line to the data by means of linear regression analysis allows an
160 easy and precise determination of the intercept value at the ordinate. This value is equal to the inverse
161 of immunoreactive fraction of radiolabeled monoclonal antibodies.

162 The specificity of ⁸⁹Zr-DFO-CTX for human and murine EGFR antigen was tested using A431 cells
163 and the mouse glioma cells GL261 that overexpresses murine EGFR. For each cell line, the incubation
164 was performed either at a low concentration of ⁸⁹Zr-DFO-CTX alone (1 µg for 1x10⁶ of cells, 1 µCi)
165 or in the co-presence of a large excess of CTX (1000 µg for 1x10⁶ of cells, 1 µCi)

166 **6. Animal experiments.**

167 Animal experiments have been performed using five-week old male *nu/nu* mice. They were conducted
168 according to the European directive 2010/63/EU and its transposition in the French law (Décret n°
169 2013-118). Animal experiments were conducted at the imaging facility CEA-SHFJ (authorization
170 D91-471-105/ethics committee n°44). Mice were housed in standard conditions (microisolator
171 polycarbonate cages, aspen wood as bedding material, 4 mice in each cage, room temperature 22°C,
172 humidity 40%) under a regular 12-hour dark/light cycle. Animals were fed with standard nutritional
173 cubes. Food and water were available *ad libitum*. Mice were provided with clean shredded papers or
174 cotton square nestles as nesting materials, and polycarbonate cottages and wooden sticks as
175 environmental enrichments. Animals experiments were performed under isoflurane anesthesia (2.5 –
176 3% isoflurane in oxygen for induction and 1 – 2 % isoflurane in oxygen for maintenance. The level of
177 oxygen flow that we used was in the range of 1.5 – 2 l.m⁻¹). In total, 32 mice were used for this study.
178 Mice were separated randomly into different groups. More details are described in the study design
179 ⁸⁹Zr-DFO-CTX PET experiments. Experimenters were blinded with mice's identity during
180 administration and imaging procedures.

181 **7. *In vivo* stability of ⁸⁹Zr-DFO-CTX.**

182 Blood samples were obtained from the tail vein 1 h and 48 h after *iv* injection of ⁸⁹Zr-DFO-CTX or left
183 ventricle via cardiac puncture 7 days after *iv* injection of ⁸⁹Zr-DFO-CTX. They were immediately
184 centrifuged at 2300g for 15 min at 4 °C. During the whole process, mice were kept under anesthesia or
185 deep terminal anesthesia (for cardiac puncture). Radioactivity of the supernatant was immediately
186 resolved by ITLC allowing separating ⁸⁹Zr-DFO-CTX from ⁸⁹Zr or ⁸⁹Zr-DFO. The experiments were
187 done in triplicate.

188 **8. FUS-induced BBB permeabilization.**

189 Permeabilization of the BBB was achieved using a focused transducer (active diameter 25 mm, focal
190 depth 20 mm, Imasonic, Voray sur l'Ognon, France) centered at 1.5 MHz connected to a single-
191 channel programmable generator (Image Guided Therapy, Pessac, France). The transducer has an axial
192 and a lateral resolution of 5 mm and 1 mm, respectively. The beam profile was measured using a
193 calibrated hydrophone (HGL-200, Onda Corporation, CA, USA). Reported values corresponded to the
194 width and the length at 6 dB. The transducer was mounted on a motorized XYZ-axis stage and
195 positioned above the mouse head maintained under anesthesia. The device was coupled to the mouse
196 skull using a latex balloon filled with deionized and degassed water and coupling gel. The distance
197 between the transducer and the skull was adjusted by the displacement of the motorized axis (Z) and
198 the filling of the balloon in order to get the center of the brain at the focal distance (i.e., 20 mm).
199 Typically, for transcranial BBB opening in human, a lower frequency (<0.4 MHz) is chosen to limit
200 the attenuation of the skull. In that case, the axial resolution is about few cm while lateral resolution is
201 about few mm. The clinical transfer of FUS-mediated BBB opening for GBM therapy is currently
202 under investigation.

203 A 50 µL bolus of microbubbles (SonoVue®, Bracco, Milan, Italy, 1.5×10^8 microbubbles/mL) were
204 intravenously administered in the tail vein. A mechanical zig-zag shaped scan (XY-axis) was
205 synchronized to the generator output in order to induce a wide BBB opening covering a square region
206 in the middle of the brain of 6 mm x 6 mm in order to open a large area of the mouse brain including
207 the two hemispheres but excluding the cerebellum. Motor speed was set to 10mm.s⁻¹ and the time
208 trajectory was 5.1 s. Ultrasonic waves were continuously transmitted at 1.5 MHz during the transducer
209 translation. However, to avoid an excessive deposit of ultrasound energy at a focal spot, FUS was
210 briefly turned off during the changes of direction in the mechanical scan. This results in a final duty
211 cycle of 69%. The sequence was repeated 25 times for a total exposure of 127 s. The transmitted in
212 situ peak negative pressure in the mouse brain is estimated to be 430 kPa (520 kPa in deionized water)
213 considering a skull attenuation of 18% (average value of the skull attenuation measured in Choi et al
214 [24]) at 1.5 MHz.

215 This protocol was optimized for C57BL/6 mice in a previous study. The FUS safety on nude mice was
216 also assessed during this study.[25] Here, we first validated the acoustic parameters on 6 nude mice
217 using contrast enhanced MRI as biomarker of BBB integrity. With this aim, mice were iv injected (tail
218 vein) with 100µL of gadoterate (about 1.66mmol/kg, Dotarem, Guerbet, France) right after sonication
219 ended. Mice were immediately placed inside a small animal 7T MRI (Bruker, Germany) and T1-
220 weighted images were acquired according to following parameters: Multi Slice Multi Echoes, TE 5
221 ms, TR 300 ms, resolution 0.15x0.15x0.4 mm³, matrix size 128x128x20, 8 averages, total acquisition
222 time = 5 min. Effective BBB disruption was confirmed by the enhancement of the T1-weighted signal
223 due to gadolinium leakage within the square shaped sonicated region. No side effect (edema, bleeding)
224 were observed and the distribution of gadolinium was homogeneous in the targeted area.
225

226 **9. Study design of ⁸⁹Zr-DFO-CTX PET experiments.**

227 Four groups of mice (Nu/Nu mice, 5 weeks, male) were studied: (i) the sham group, with no FUS
228 BBB opening and microdose (10 µg/mouse) of ⁸⁹Zr-DFO-CTX (high specific activity, i.e the ratio
229 between radioactivity and injected mass of mAb). This sham group was used to assess the brain
230 kinetics of ⁸⁹Zr-DFO-CTX when the BBB is intact. These animals received the microbubble injection
231 and a sham FUS protocol with zero ultrasound amplitude. (ii) FUS group. Permeabilization of the
232 BBB was achieved with the validated FUS protocol and microdose of ⁸⁹Zr-DFO-CTX (high specific
233 activity, 10 µg/mouse) injected 10 min after FUS. The tracer was injected after FUS protocol in order
234 to obtain the entire kinetics of mAb from the time of injection till 7 days after injection. (iii) The
235 pharmacologic dose (PD, 1mg of unlabeled CTX per mouse was added) group, without FUS BBB
236 opening and low specific activity. This group allows determining the impact of CTX diffusion into the
237 healthy brain with high dose of CTX mimicking the pharmacologic situation. (iv) FUS pharmacologic
238 dose group with FUS-induced BBB permeabilization co-injected with a target- pharmacologic dose
239 ⁸⁹Zr-DFO-CTX (low specific activity, 1mg per mouse) (Fig. S11.).
240

241 **10. PET acquisition.**

242 PET acquisitions were performed using a Siemens Inveon small animal PET scanner associated to a
243 CT scanner (Knoxville, TN, USA). The catheter inserted in the tail vein for injection of microbubbles
244 (50 μ L) was then used for injection of ^{89}Zr -DFO-CTX. The injection of ^{89}Zr -DFO-CTX (in <150 μ L
245 saline solution) was performed 10 min after FUS under the PET scanner. All groups received similar
246 doses of radiolabeled ^{89}Zr -DFO-CTX (0.24 ± 0.07 MBq/g). A 60 min dynamic PET acquisition was
247 first performed immediately after injection of the ^{89}Zr -DFO-CTX. Then, at selected time points after
248 injection (4h, 24h, 48h, 72h and 7 days after the injection), mice were anesthetized and installed under
249 the PET scanner for a 30 minute static PET acquisition. All the mice (n=8) were imaged at all the
250 time points, except for the FUS groups (microdose and pharmacologic dose) at 4 hours (n=6).

251

252 **11. PET images reconstruction and analysis.**

253 The spatial resolution of the PET scanner is ~ 1.5 mm (FWHM). PET images were reconstructed using
254 a 2D-OSEM iterative algorithm. Volumes of interest (VOI) were delineated manually based on CT
255 images on sonicated/non-sonicated brain regions and the heart-blood pool on summed PET images
256 using PMOD software (Version 3.9, Switzerland) and Carimas (Turku PET Centre, Finland). All the
257 images and extracted data were corrected according to the half-life of ^{89}Zr in order to obtain the
258 accurate uptake in each organ. Time-activity curves (TACs) were generated from each VOI to describe
259 the kinetics of ^{89}Zr -DFO-CTX. The activities were expressed as the percentage of injected dose per
260 volume (%ID/cc). The area under the TACs (AUC) from time 0 to 60 min for the dynamic scan was
261 calculated to describe the organ exposure to CTX (GraphPad Prism). The total volume of distribution
262 (V_T , mL.cm $^{-3}$) defined as the ratio of the radioligand concentration in the tissue of interest to that in the
263 blood at equilibrium was estimated using the Logan graphical method.[26] V_T encompasses both the
264 BBB permeation and the total binding of ^{89}Zr -DFO-CTX to the brain. Estimation of V_T was not
265 possible in two mice that show large movement due to respiration, which made it impossible to get
266 accurate blood function.

267 **12. Integration Plot Analysis.**

268 Integration plot analysis was used to graphically estimate the uptake transfer of ^{89}Zr -DFO-CTX from
269 blood to plasma ($k_{\text{uptake,brain}}$) across the BBB, as previously described.[27] The uptake from blood to
270 brain was calculated by the integration plot method using the portion of time profile of the
271 radioactivity during which brain radiotracer excretion from the blood or other organs was negligible
272 (between 15s to 3min). Similar to V_T estimation, the $k_{\text{uptake,brain}}$ was not estimated in two mice that
273 present large heart movement due to respiration. Mathematical models for Logan plot and integration
274 plot were provided in more details in the Supplementary Information.

275 **13. Statistics.**

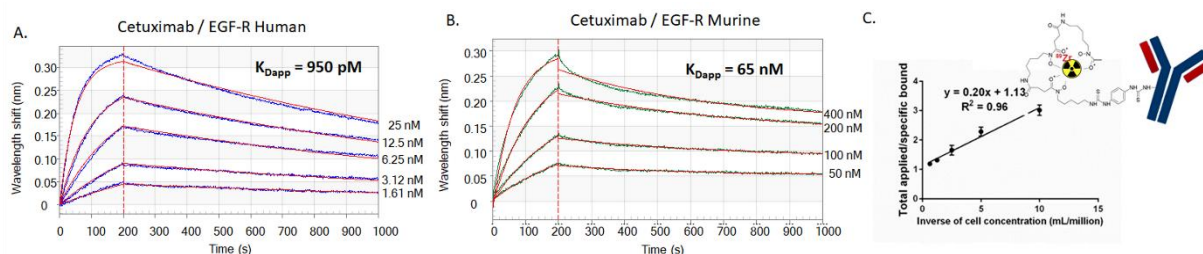
276 All data are presented as mean \pm standard deviation. The statistical analyses were performed using
277 GraphPad Prism software (Graph Pad software Inc., San Diego, USA). Comparisons of PET data
278 between FUS and Non FUS over time were performed using a one-way ANOVA. Changes at the 95%
279 confidence level ($P < 0.05$) were qualified as statistically significant.

280 **III. RESULTS**

281 **1. The relevance of using CTX in a mouse model for assessing the effect of FUS to increase**
282 **mAb diffusion through BBB.**

283 Correct interpretation of the brain kinetics of a mAb requires information regarding its specific and/or
284 nonspecific binding to target tissues. CTX is a chimeric monoclonal antibody where the variable
285 regions of immunoglobulins have a murine origin [28]. Previous studies suggested a low binding on
286 murine EGFR, specifically with NMRI mouse by competitive binding assays [29]. However, binding
287 kinetic constants in a direct binding assay remained to be determined. K_D of CTX for human EGFR
288 was measured at 950 pM for human EGFR, approximately ~ 70 times higher than for murine EGFR
289 ($K_D = 65$ nM) (Fig. 1A and B). Binding assays on human (A431) and mouse (GL261) cells expressing
290 high level of EGFR were also conducted using the radiolabeled compound ^{89}Zr -DFO-CTX. Before the

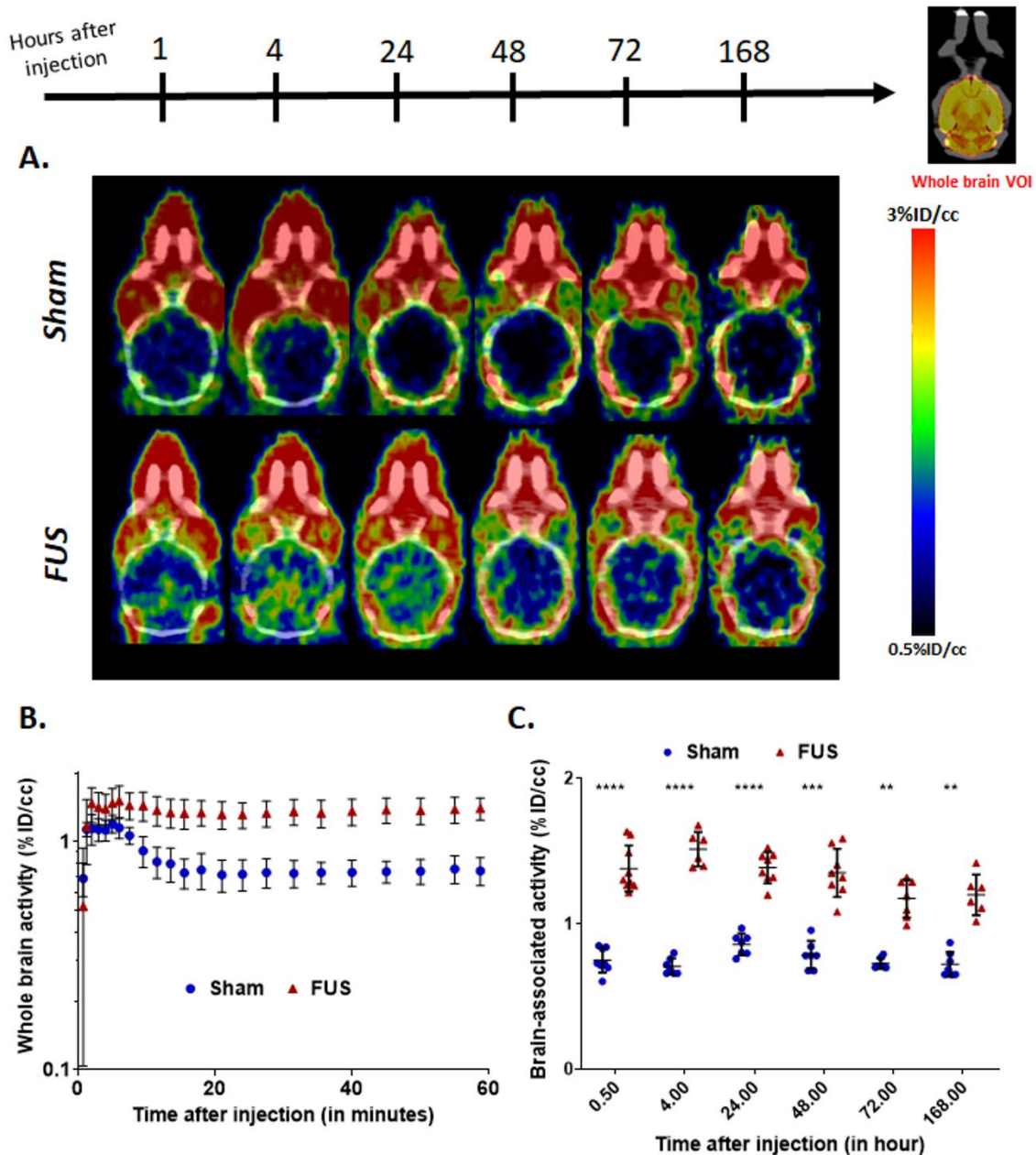
291 assays, to control that the radiolabeling process does not strongly affect the affinity of CTX for human
 292 EGFR, the immunoreactive fraction of ^{89}Zr -DFO-CTX was also measured on A431 cells at infinite
 293 antigen excess. The immunoreactive fraction was determined to be close to 100% (Fig. 1C). The
 294 results of binding assays also showed low uptake of CTX for mouse GL261 cells and high uptake for
 295 human A431 cells (Fig. SI2). Moreover, the binding of CTX to A431 cells is displaceable whereas the
 296 binding of CTX to GL261 cells is non-displaceable.



297
 298 **Figure 1. The kinetic rate constants of CTX for human EGFR and murine EGFR.** Kinetic rate
 299 constants of interaction between CTX and human EGFR ectodomain (A) or murine EGFR ectodomain
 300 (B) were determined using BioLayer Interferometry. C. The immunoreactive fraction of ^{89}Zr -DFO-
 301 CTX was measured by performing a linear regression analysis of a plot of (total/bound) activity versus
 302 $(1/[\text{concentration of cells}])$ yielding the immunoreactive fraction determined as $1/y$ -intercept.

303
 304 **2. FUS enabled the BBB permeation of ^{89}Zr -DFO-CTX.**

305 Firstly, T_1 -weighted MRI after the injection of gadoterate was performed to validate the procedure for
 306 FUS-induced permeabilization of the BBB (Fig. SI3 A.). The result showed a persistent contrast
 307 enhancement in the sonicated brain area.



308

309 **Figure 2. Brain kinetics of ^{89}Zr -DFO-CTX after FUS-induced BBB permeabilization. (A)**
 310 Representative PET images obtained from the same mouse at different time points (1 h, 4 h, 24 h, 48
 311 h, 72 h and 168 h after injection) for the *sham* (top) and the FUS (bottom) groups. Time-activity
 312 curves (TACs) in the whole brain of ^{89}Zr -DFO-CTX injected to sham group (blue) and FUS group
 313 (red) group during 60 min (B) and up to 168 h (C) (n=8 for each group). Data were extracted from a
 314 whole brain volume of interest (VOI, represented by a yellow color zone inside the skull). Statistical
 315 significance was determined using unpaired *t* tests with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****
 316 $p < 0.0001$. All data are represented as mean \pm SD. No statistical significance was observed for the
 317 sham group, at any time after injection. Sagittal images are in FigSI3.B.

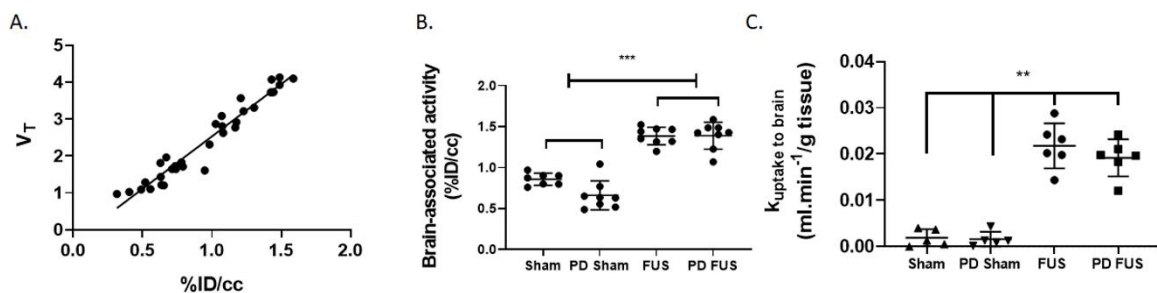
318 Then, the same protocol of FUS was applied before the injection of ^{89}Zr -DFO-CTX. FUS protocol
 319 does not have an impact on the overall distribution of ^{89}Zr -DFO-CTX except on the brain area where
 320 FUS was applied (Fig. SI9). We have found that BBB permeabilization enabled by FUS significantly
 321 enhanced the brain uptake of ^{89}Zr -DFO-CTX (Fig. 2A and B). The initial accumulation of ^{89}Zr -DFO-
 322 CTX in the brains of FUS group was higher than in the sham group as early as 15 min after the
 323 injection of labeled compound. Maximal brain concentration (C_{max}) occurred at 4 hours after injection.

324 The maximal brain concentration of ^{89}Zr -CTX in the brain without FUS was 0.70 ± 0.06 %ID/cc,
 325 which corresponds to the vascular content. The brain concentration of ^{89}Zr -CTX in FUS-treated
 326 animals was significantly higher and maximal after 4h (1.51 ± 0.12 %ID/cc, $p < 0.001$). The activity at
 327 the plateau after BBB permeabilization was almost two times higher than the one obtained in the *sham*
 328 group. The difference between two groups remained significant even up to 168 h post injection (Fig.
 329 2C).

330 On the other hand, metabolic stability of ^{89}Zr -DFO-CTX was assessed in plasma samples obtained at 1
 331 hour, 48 h and 168 h post FUS. No release of DFO- ^{89}Zr , free ^{89}Zr , or other radiolabeled fragments
 332 could be observed (Fig. SI4).

333 3. Modeling ^{89}Zr -DFO-CTX brain kinetics to elucidate the impact of BBB disruption on mAb 334 delivery into the brain.

335 Besides giving the concentration of radioactivity (%ID/cc) in tissue, our dynamic PET images also
 336 allow estimating the total volume of distribution (V_T) and the uptake kinetic constant (k_{uptake}). We have
 337 found that the concentration of radioactivity in the brain at 24h post-injection was significantly
 338 correlated with the V_T estimated in the first 60 min post injection from dynamic data ($P < 0.0001$; $r^2 =$
 339 0.94). This correlation included animals of the sham, pharmacologic dose sham, FUS and
 340 pharmacologic dose FUS groups (Fig.3A). A significant correlation was also observed between brain
 341 V_T and brain AUC ($P < 0.0001$, $r^2 = 0.92$) as shown in (Fig. SI5). Both parameters were estimated from
 342 dynamic data obtained from 0 to 60 min post injection.



343

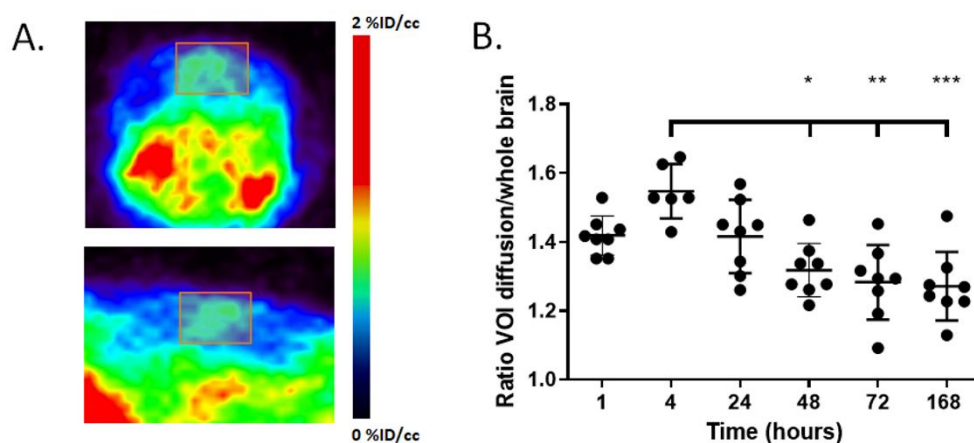
344 **Figure 3. Comparison of PET imaging quantification between different groups and**
 345 **pharmacokinetics calculation.** A. Correlation between V_T and the concentration of activity (%ID/cc)
 346 at 24 h post-injection. B. Concentration of activity in the brain 24 hours after injection in different
 347 groups (sham, pharmacologic dose (PD) sham, FUS and pharmacologic dose (PD) FUS). C. Brain
 348 uptake transfer (k_{uptake}) of ^{89}Zr -DFO-CTX in different groups. ** $P < 0.01$, $n = 8$ mice per group.

349 The assumption of equivalent nonspecific binding is usually evaluated *in vivo* with the displacement of
 350 radiotracer binding by high doses of a nonradioactive drug, called a pharmacologic dose [30]. Thus, to
 351 confirm that specific binding does not account for ^{89}Zr -DFO-CTX brain distribution, we compared the
 352 brain kinetics of microdose ^{89}Zr -DFO-CTX (low amount of CTX included in the radiotracer injection
 353 dose) with a pharmacologic dose (radiolabeled CTX co-injected with a large amount of unlabeled
 354 CTX) in different conditions (Sham and FUS). No difference on the tracer uptake was observed
 355 between the microdose and the pharmacologic dose condition (sham: 0.85 ± 0.07 % ID/cc vs
 356 pharmacologic dose: 0.67 ± 0.18 % ID/cc and FUS: 1.38 ± 0.11 % ID/cc vs FUS pharmacologic dose:
 357 1.39 ± 0.16 %ID/cc). Fig 3.B. The V_T of ^{89}Zr -DFO-CTX in brains of different groups, derived from
 358 the dynamic PET imaging (first hour after injection) corroborated this result (Fig SI6). Since the ^{89}Zr -
 359 DFO-CTX uptake in the whole brain was not affected by the pharmacologic dose displacement assay,
 360 ^{89}Zr -DFO-CTX was not impacted by murine EGFR expression. To confirm this *in vivo* observation,
 361 on nu/nu brain mice frozen slice, *in vitro*, ^{89}Zr -DFO-CTX and fluorescent CTX were incubated.
 362 Neither radioactive signal nor fluorescent signal were observed (FigSI10).

363 The rate constant for the transfer of radiotracer from the blood to the brain (k_{uptake}), which was
 364 calculated based on the integration plot analysis from the initial brain uptake of the radioactivity,
 365 shortly after the injection, was significantly higher in the FUS group ($13 \times 10^{-3} \pm 6 \times 10^{-3} \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$)
 366 than in the sham group ($29.3 \times 10^{-6} \pm 15 \times 10^{-6} \text{ ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$). No significant difference of transfer rate
 367 was observed between pharmacologic dose and the microdose groups.

368 4. Slow diffusion of ^{89}Zr -DFO-CTX within the brain parenchyma.

369 Longitudinal imaging study was performed for several days after FUS-induced BBB permeabilization
 370 to investigate the diffusion of ^{89}Zr -DFO-CTX from the sonicated area to the brain parenchyma. A
 371 threshold was applied into the PET images to suppress the background and facilitate the determination
 372 of the volume of ^{89}Zr -DFO-CTX accumulation in the brain after BBB permeabilization (Fig. 4A). The
 373 threshold that was chosen corresponds to the mean of the concentration of activity (%ID/cc) in the
 374 cerebellum plus two times the standard deviation. The cerebellum region was used as the region of
 375 reference since no FUS was applied on this region. Indeed, the uptake in the cerebellum remains
 376 constant over time (Fig. SI7). In order to quantify the diffusion of mAbs over time, the activity of the
 377 defined (by the threshold) volume was compared to the activity of the whole brain volume (Fig. 4B).



378
 379 **Figure 4.** ^{89}Zr -DFO-CTX diffusion within the brain after FUS-mediated BBB opening. **A.** PET images
 380 showing the delineation of radiolabeled CTX four hours after FUS-induced BBB permeabilization.
 381 The box in orange indicate the area of the threshold applied to delimitate the brain parenchyma where
 382 was applied the FUS **B.** Ratios between the activities onto the VOI defined at four hours and the whole
 383 brain activity at different times. Significant differences were observed between 4 hours and 48, 72 and
 384 168h (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 8$ mice per group).
 385

386 In each individual animal, the elimination half-life of ^{89}Zr -DFO-CTX from the sonicated brain tissue
 387 was calculated by fitting the time decay of the activity with an exponential function. Brain $T_{1/2}$ was
 388 determined to be in average equal to 21.4 ± 1.7 h. Meanwhile, the elimination half-life from blood was
 389 calculated using the blood activity over time $T_{1/2} = 5.7 \pm 0.4$ h. In sham animals, the elimination half-life
 390 of ^{89}Zr -DFO-CTX from brain was 5.01 ± 0.7 h, consistent with the blood $T_{1/2} = 6.3 \pm 0.2$ h.

391 After FUS was applied, the amount of ^{89}Zr -DFO-CTX in the brain remained stable, with a slight
 392 decrease after several days. The ratio of radioactivity measured in the sonicated region to the whole
 393 brain was maximal at 4 hours after the injection and then slowly decreased down to 1.27 ± 0.10 , 168
 394 hours after the injection. This suggests a limited and slow diffusion of ^{89}Zr -DFO-CTX from the
 395 sonicated area to the surrounding brain region and a limited clearance of ^{89}Zr -DFO-CTX from the
 396 targeted tissue, allowing for high and localized exposure.
 397

398 IV. DISCUSSION

399 EGFR is one of the major target for cancer therapy in a large variety of cancers [31]. Despite some
400 promising studies proposing EGFR-targeting therapies such as small molecule tyrosine kinase
401 inhibitors or mAbs with CTX, the potential of these agents against glioblastoma has been unfulfilled
402 [32]. One of the reasons for the resistance of glioblastoma cells to treatments might be the limited
403 diffusion of molecules in general and biologics in particular across the BBB that has been
404 underestimated. Till recently, it has been taken for granted that the presence of glioblastoma tumors
405 induced an obvious physical disruption of the BTB in patients [33,34]. To our knowledge no clinical
406 trials have explored or even tried to predict the ability of mAb to reach the tumors as a factor of
407 response in patients with glioblastoma. A limited number of groups have indirectly suggested a link
408 between mAb efficacy and BBB integrity in preclinical studies [9,35]. In this context, FUS has
409 emerged as a clinical mean to increase the permeability across the BBB to therapeutics including
410 mAbs. From the initial studies where high-intensity FUS was applied to increase the permeability of
411 mAb into subcutaneous tumors,[36] newer strategies have evolved where the combination with
412 microbubbles administration help reduce the required intensity of FUS and induce a greater
413 permeabilization capacity. Such mild approach might be safer, more suitable for delicate tissues and
414 appears to be efficient for BBB. A total of six trials have been or are currently conducted on GBM
415 patients using a variety of devices including SonoCloud® (CarThera), ExAblate® (InSightec), and
416 NaviFUS® (NaviFUS cooperation), both with and without chemotherapy regimens (for clinical
417 safety), such as carboplatin, doxorubicin and temozolomide[37]. However, only few noninvasive
418 methods have been explored to characterize FUS-enhanced mAb delivery through the BBB in a
419 translational perspective. Fluorescent antibody-based ligands have been developed to characterize
420 FUS-mediated mAb delivery and demonstrate that this technique is valuable to monitor antibody
421 delivery.[38] However, fluorescence imaging is difficult to translate into clinic due to the poor tissue
422 penetration of light. In this aspect, PET imaging is superior to fluorescence imaging thanks to much
423 higher penetrability of gamma ray. Hence, it offers a sensitive, dynamic and quantitative imaging
424 modality to investigate the distribution of mAb in the whole-brain parenchyma after FUS-induced
425 permeabilization of the BBB. Therefore, PET imaging has more potential to be transferred to clinical
426 trials in a rather straightforward manner. Several groups are currently using this so-called immunoPET
427 technique for peripheral tumor imaging in clinical trials [39,40]. ImmunoPET is expected to become
428 widespread in the future due to the increase of immunotherapy strategies around the world. This
429 molecular imaging technique could improve drastically the efficacy of treatment by monitoring
430 quantitatively the efficacy of drug delivery as well as to visualize the distribution of predictive
431 biomarkers in deep-seated organs and/or in a large area, or even in whole-body scale.

432 It is to our knowledge the first study to clarify the kinetic impact of FUS on the BBB crossing and
433 diffusion of mAb in the brain parenchyma. The use of translational imaging paves the way for the use
434 of PET with radiolabeled mAb (^{89}Zr -DFO-CTX in this particular case) to address the
435 neuropharmacokinetic correlation of the efficacy of mAbs in brain tumors. This approach will be
436 useful to provide the proof-of-concept on the potential of FUS to enable molecularly targeted
437 immunotherapy against CNS tumors with intact BBB.

438 According to the Allen Brain Atlas and to Estrada *et al.*, low EGFR expression was found in the brain
439 of healthy mice [41]. Endogenous expression of murine EGFR in brain is therefore not likely to
440 account for ^{89}Zr -DFO-CTX binding to the brain. We have shown that CTX and ^{89}Zr -DFO-CTX have
441 low affinity for the murine epitope, that have been already described by different groups [42].
442 Moreover, the displacement in vivo assay with the pharmacological dose prove the non-specificity of
443 ^{89}Zr -DFO-CTX for murine EGFR expression. ^{89}Zr -DFO-CTX kinetics therefore predominantly reflect
444 the mechanical uptake of the compound across BBB rather than its binding.

445 The BBB is a unique and highly selective barrier composed of cerebral endothelial cells tightly
446 connected to each other by tight junctions and adherent junctions [43]. Intact BBB limits the brain
447 penetration of antibodies from the systemic circulation [35]. Typically, antibody concentrations are
448 1,000 times lower in the brain than in the bloodstream. Moreover, the low passage of mAb through the

449 intact BBB is a slow phenomenon [44]. ^{89}Zr -DFO-CTX PET images in *sham* animals confirmed the
450 negligible permeation of CTX across the intact BBB. After FUS treatment, we clearly show a drastic
451 increase in the uptake of ^{89}Zr -DFO-CTX in the sonicated brain area (two times compared to the intact
452 BBB brains in the sham group). The accumulation was maximum during the first hours right after the
453 FUS opening. The BBB closure is a progressive process. Small particles ($< 1\text{nm}$) are able to pass
454 through the BBB for hours while the passage of large particles ($>50\text{ nm}$) is limited to few seconds.
455 Conti et al. have presented a mathematical model able to predict the evolution time of drugs
456 concentration in the function of their size after BBB permeabilization by FUS [45]. According to this
457 study, mAbs (around 150 kDa) should be able to cross the BBB only during the first 15 minutes after
458 FUS was applied since the disruption of tight junction is only transient. This model assumes that
459 molecular size is the only parameter directing BBB crossing while other properties can explain
460 deviations from this model: molecule anisotropy, lipophilicity, surface charge, active transport [46].
461 Furthermore, even if the mean tight junction size is expected to be smaller than CTX size very fast
462 after US, a limited number of junctions will remain open for this size for much longer time and could
463 explain our results since CTX circulates for a long time.

464 The transfer rate characterizing the passage of mAb through the BBB was boosted up to $2\text{ mL}\cdot\text{min}^{-1}\cdot 100\text{gr}^{-1}$
465 right after BBB opening from almost zero without BBB opening. Making the best with the
466 long physical decay of ^{89}Zr , the elimination half-life of ^{89}Zr -DFO-CTX from the sonicated brain tissue
467 ($T_{1/2} = 21.4\pm 1.7\text{h}$) was significantly different from the elimination half-life from blood ($T_{1/2} =$
468 $5.7\pm 0.4\text{h}$). Elimination from the sonicated brain was significantly slower, which confirmed the
469 retention of ^{89}Zr -DFO-CTX in the targeted tissue. In *sham* animals, the elimination half-life from the
470 blood and from the brain were similar, thus suggesting that the intact BBB brain radioactivity mainly
471 reflected the vascular content of the brain compartment.

472 The brain concentration of ^{89}Zr -DFO-CTX (in %ID/cc) are tightly correlated to the brain V_T , which
473 considers the blood radioactivity kinetics. The V_T of ^{89}Zr -DFO-CTX in the brain therefore provides a
474 more precise parameter to describe the distribution of the mAb to the brain parenchyma over time.
475 This strong correlation between V_T and brain activity suggest that FUS does not affect the blood
476 kinetics and metabolism of ^{89}Zr -DFO-CTX as well as the vascular content of the brain compartment.

477 Two different concentrations of CTX, $10\text{ }\mu\text{g}/\text{mouse}$ and $1\text{ mg}/\text{mouse}$ were tested for every condition
478 (without and with BBB opening). We observed no difference in the overall uptake of ^{89}Zr -DFO-CTX
479 by the brain between the low dose ($10\text{ }\mu\text{g}/\text{mouse}$) and the pharmacologic dose ($1\text{ mg}/\text{mouse}$) whatever
480 the status of BBB integrity was. CTX has low affinity for mouse EGFR that suggests a limited binding
481 of ^{89}Zr -DFO-CTX to the mouse organs. This observation suggests that the uptake did not depend on
482 the ^{89}Zr -DFO-CTX / CTX ratio in the blood-pool. It was confirmed by the transfer rate which has
483 neither been modified at the low dose nor at the pharmacologic dose. The BBB is, therefore, the main
484 determinant of the brain kinetics of CTX. The brain distribution of mAb therefore reflects the
485 extravasation and elimination processes driven by the convective lymph flow to and from the tissue,
486 and the vascular and interstitial reflection coefficients. This observation has been suggested by other
487 groups [47,48]. The microdose ^{89}Zr -DFO-CTX therefore predicts the pharmacologic situation and can
488 be safely used in clinical trials to study the brain kinetic of CTX.

489 We show that the amount of ^{89}Zr -DFO-CTX delivered to the sonicated area only slowly diffuses
490 throughout the brain parenchyma, suggesting appreciable accumulation of the mAb in the targeted
491 region. This result is consistent with the expected typical passive diffusion of CTX in the extracellular
492 compartment according to its estimated molecular size. In the first hours after the injection, there was
493 a slight local diffusion and accumulation of labeled CTX right below the center zone where FUS was
494 applied. After 24 hours, there was a slow but significant release of the radioactivity from the sonicated
495 area to the surrounding brain tissue demonstrated by a gradual decrease of the ratio between the
496 activity in the locally-diffused VOI and the one in the whole brain. **Moreover, the CTX seems to be**
497 **slightly decreasing overtime taking into consideration the global brain uptake. This CTX elimination**
498 **may be explained by the lymph turnover process involved in the brain [49].** This means that once BBB
499 integrity is restored, CTX action will be restricted to the FUS area where it entered brain parenchyma.

500 Limited penetration and the associated steep concentration profiles of mAb in function of distance into
501 the brain after local delivery were already well documented [50]. Wolak et al. have shown that IgG
502 antibody diffusion in brain extracellular space is limited compared to in free solution by nearly one
503 order of magnitude. The extracellular distribution in brain tissue is significantly hindered [50]. In
504 addition, Silburt et al. have shown that FUS perturbation seems to have a little influence on biologic
505 flow through the brain. But this effect still remains limited without inducing a large change in the
506 diffusion properties of the brain cortical space [51].
507

508 **V. CONCLUSION**

509 Our results show the impact of FUS-induced transient BBB permeation on the delivery of CTX across
510 the BBB and subsequent brain exposure. To this end, we used pharmacokinetic in vivo imaging
511 making the best from quantitative capacity of PET and long half-life ⁸⁹Zr labeled CTX. FUS induced a
512 drastic increase in the penetration of CTX to the brain followed by a low diffusion of the mAb within
513 the brain parenchyma. Our results show the relevance for FUS technology to enable sufficient and
514 targeted exposure of the brain tissue to mAbs. In this context, FUS may ultimately provide a relevant
515 and realistic technique to enable CNS targeted immunotherapy, especially with anti-EGFR treatment.
516 This complete study is the essential first step to further discriminate between BTB and BBB to
517 improve FUS mAb delivery in GBM environment. Further experiments are needed to test whether this
518 technique can significantly improve the delivery of ⁸⁹Zr-DFO-CTX in GBM orthotopic models with
519 different levels of BTB disruption and BBB integrity. Malignant tissue might present, to some extent,
520 similarity to healthy brains as well as some disparity in terms of physiology and biology. In view of
521 such a broad prospective, this work hopefully provides another strong evidence to pave the way for
522 more investigations into this exciting field.
523

524 **COMPETING INTERESTS**

525 The authors have declared that no competing interest exists

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528 ARC.

529 **ABBREVIATIONS**

530 EGFR: epidermal growth factor receptor; CTX: cetuximab; mAb: monoclonal antibody; BBB: blood
531 brain barrier; CNS: central nervous system; BTB: blood-tumor barrier; FUS: focused ultrasound; MRI:
532 magnetic resonance imaging; PET: positron emission tomography; DFO: Deferoxamine; iTLC: instant
533 thin- layer chromatography, ⁸⁹Zr: Zirconium-89Zr.
534
535

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