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EVALUATION OF ANTIVASCULAR COMBRETASTATIN A4P EFFICACY BY USING SUPERSONIC SHEAR IMAGING TECHNIQUE OF ECTOPIC COLON CARCINOMA CT26

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25 **Abstract**

26 A recent ultrasound imaging technique, called shear wave elastography, showed its
27 ability to image and quantify mechanical properties of biological tissues, such as prostate or
28 liver. In the present study this technique was used to evaluate the relation between tumor
29 growth, stiffness and tumor reduction upon treatment with combretastatin (CA4P) in allografted
30 colon tumor CT26, in mice. During twelve days, CT26 tumor growth (n=52) was imaged by
31 ultrasound, and shear modulus was quantified, showing a good correlation between tumor
32 volume and stiffness ($r = 0.59$). At day 12, the treatment was initiated and monitored every day
33 during 4 days. Following the treatment, the tumor volume has decreased, still the elasticity
34 parameter of the tumor volume was steady throughout the treatment. After segmentation using
35 the shear modulus map, a detailed analysis showed a decrease in the stiffness after treatment as
36 compared to the control group. This reduction in the mechanical properties was shown to
37 correlate with tissue reorganization, in particular fibrosis and necrosis, assessed by histology.

38

39 Keywords: shear wave elastography, ultrafast ultrasound imaging, antivascular treatment, mice
40 colon carcinoma model, histological parameters.

41

42 **Introduction**

43 Shear wave elastography (SWE) is an ultrasound imaging technique that allows imaging
44 and quantification of the mechanical properties of soft tissues (Sarvazyan et al., 1998). Multiple
45 variations of this technique have been developed throughout the years (Gennisson et al., 2013;
46 Doyley et al., 2014) such as Shear wave Dispersion Ultrasonic Velocity (Chen et al., 2002),
47 Acoustic Radiation Force Impulse (Nightingale et al., 2003) or Supersonic Shear Imaging (SSI)
48 (Bercoff et al., 2004) which quantify or estimate the tissue stiffness. Stiffness of the human
49 tissue is of great interest to physicians, since it relates to the palpation which is a basic step of
50 the clinical exam. Few studies have shown that tumor stiffness quantification and mapping
51 improves the physician diagnosis (Berg et al., 2012; Itoh et al., 2006; Lee et al., 2011). SWE
52 was also applied in other organs such as the liver (Palmeri et al., 2008; Deffieux et al., 2015)
53 and the prostate (Correas et al., 2013), showing the interest of quantifying the stiffness to better
54 understand cancer pathologies.

55 Regarding cancer treatment, conventional ultrasound imaging is useful to quantify
56 morphological tumor size evolution. However, it would be of great interest to add new imaging
57 parameters that allow both early evaluation of the treatment and longitudinal monitoring. In this
58 context, shear wave elastography can be a complementary technique providing informations on
59 tissue viscoelastic properties, as an adjunct to conventional morphological ultrasound imaging.
60 To our knowledge only a few studies have been done using shear wave elastography to follow
61 cancer treatment. In 2013 and 2015, Evans and Athanasiou, respectively, used the SSI technique
62 to follow breast cancer chemotherapy in humans (Evans et al., 2013; Athanasiou et al., 2015).
63 [These studies concluded that tumor stiffness can be a striking factor to predict chemotherapy](#)
64 [treatment efficiency, since tumor stiffness was shown to decrease faster during treatment than](#)
65 [the tumor volume assessed by conventional ultrasound.](#)

66 **Notwithstanding**, it still remains challenging to understand the relationship between the
67 macroscopic changes and the tissue remodeling due to the treatment. Regarding histological
68 parameters, recent studies involving SWE results and breast tumor histological analysis have
69 found controverting results. Chang et al. found that mucinous cancer in humans, which is often
70 classified as softer lesions by pathologists, showed higher stiffness values than other types of
71 breast cancer (Chang et al., 2011). Evans et al. demonstrated a relationship between SWE
72 findings and histological prognostic factors (Evans et al., 2012). However, this study was not
73 explicit on the tumor pathological features underlying the stiffness phenomenon. In order to
74 better understand these issues, few studies have been conducted to characterize breast cancer at
75 different stages. In a preclinical study, Chamming's et al. linked the changes in SWE to an
76 increased fibrosis during tumor growth (Chamming's et al., 2013). Moreover, some authors
77 showed a correlation between the SWE values and immune-histochemical profiles of invasive
78 human breast cancer (Youk et al., 2013; Ganau et al., 2014). **However, treatment monitoring**
79 **has not been performed by using SWE methods**. Likewise, clinical results of SWE reported in
80 humans (Athanasiou et al., 2015), were not correlated to any histological parameters
81 (Chamming's et al., 2015).

82 The aim of this study is to investigate the link between histopathological
83 characterization and SWE measurements by using SSI techniques during antivasular treatment
84 in a murine colon carcinoma model (CT26). This model was selected based on recent histo-
85 pathological studies, which showed both well vascularized tumor and cellular adhesion
86 molecules (CAM). CAMs are important targets for anti-angiogenic therapies, involved in the
87 process of angiogenesis for tumor growth, cell migration and metastasis (Seguin et al., 2012).
88 Combretastatin A4 phosphate (CA4P) was chosen as it is a particular effective antivasular
89 drug, with an early post treatment effect (El-Emir et al., 2005). This vascular disrupting agent
90 CA4P was described to induce a necrosis at the center of the tumor and a fibrosis at the viable

91 rim in periphery of the tumor (Dark et al., 1997), where necrosis is defined by a passive,
92 accidental cell death resulting from environmental perturbations (Fink et al., 2005) and fibrosis
93 by an overgrowth, hardening and/or scarring of various tissues attributed to an excessive
94 deposition of extracellular matrix components including collagen (Wynn et al., 2008).

95 In the present study, the **efficacy** of antivasular treatment and the global stiffness were
96 evaluated with conventional US and SSI technique respectively. Histological analyses were
97 conducted at each stage to link the SWE results to changes in vascular density, cellularity and/or
98 fibrosis.

99

100 **Materials and Methods**

101 **Animal model**

102 Fifty two 8-week-old female Balb-C mice with a mean weight of 20 g (Janvier, Saint
103 Genest de l'Isle, France) were implanted with CT26 tumor fragment (3 mm³) subcutaneously
104 into the flank (n = 52) as previously described (Seguin et al., 2013). After implantation, the
105 growth of the tumor was imaged with ultrasound from day 5 to day 12. All animals received
106 animal care in compliance with the European Communities Council Directive of 2010. This
107 study was approved by the local committee for animal care (Comité d'éthique en matière
108 d'expérimentation animale n°34, Saisine 12-175, 'Paris Descartes') and received the agreement
109 number # CEEA34.JS.142.1. In the experimental protocol certain parameters were established
110 such as, weight loss higher than 20%, hostility between animals, abnormal behavior, shaggy
111 fur, etc... In these cases the animals were sacrificed and excluded from the study.

112

113 **Combretastatin A4 treatment**

114 At day 12, 13 and 14 after tumor implantation half of the mice (n=26) were injected
115 intraperitoneally with 100 mg/kg of CA4P disodium salt (Toroma Organics, Saarbrücken,

116 Germany) dissolved in sterile saline solution (10 μ l/g). The other 26 mice were used as a control
117 group and received 10 μ l/g of saline solution.

118

119 **Ultrasound (US) imaging**

120 Ultrasound imaging was performed on each day of the treatment using an ultrafast
121 ultrasound device (Aixplorer, Supersonic Imagine, Aix en Provence, France) with a framerate
122 of 20000 images/s. The images were acquired using a high-frequency ultrasound probe (central
123 frequency 15 MHz, 256 elements, 125 μ m pitch, Vermon, Tours, France). Before performing
124 the acquisitions, the mice were anesthetized using a gas mixture of isoflurane at 1.5% isoflurane
125 (Aerrane®; Baxter S.A.S, France), oxygen (0.5 l/min) and air (1 l/min) and were subsequently
126 shaved. Body temperature was monitored and controlled during the length of acquisition.

127 By using classical ultrasound imaging, B-mode, the operator localized the tumor within
128 the imaging plane in real time by placing the probe on the anaesthetized mice. Once the probe
129 was set up, ultrasound B-mode imaging was performed in order to define tumor morphology.
130 This acquisition involved 2 perpendicular B-mode imaging planes (transverse and sagittal). [The
131 probe was oriented until the tumor size was maximized, for each acquisition at different time
132 points \(Seguin et al., 2013\).](#)

133 Thus, three diameters (two in the axial and one in the sagittal planes) were recorded,
134 giving access to volume quantification according to the following ellipsoid formula
135 $(\pi/6) \times L \times W \times H$, where H is the height, L is the length and W is the width in centimeters.
136 (Cheung et al., 2005).

137 The SSI technique was used to measure the mean tumor stiffness under the assumption
138 that the tissue is isotropic, locally homogeneous and purely elastic (Bercoff et al., 2004).
139 Stiffness values were directly reported by the commercial system. Elastography images were
140 then acquired daily during the treatment of the animals with the Combretastatin A4 P agent in

141 the central-sagittal and central-transversal planes of the tumors. In order to adjust the stiffness
142 contrast on the image, the colorbar of the Aixplorer device was fixed between 0 and 40 kPa,
143 with the pixels in blue and red representing the lowest and highest elasticity values,
144 respectively. The clinical interface of the Aixplorer provides a Young's modulus value under a
145 soft tissue hypothesis. Nevertheless in this paper we present the results of the Shear Modulus
146 which is 1/3 of the Young's Modulus, between 0 and 13.3 kPa (Sarvazyan et al., 1998). The
147 regions of interest (ROIs) were placed so as to cover the maximum surface of the tumor within
148 the imaging plane. These ROIs (called Qbox™ in the Aixplorer device) were circular as defined
149 in the clinical interface. An average of 2-3 region of interest (ROI) measurements in 6 images,
150 taken on 2 different planes, was calculated to obtain the value of the global elasticity for each
151 animal on each day. In the aim of refining analysis, B-mode images were then manually
152 segmented by using a homemade software developed in Matlab (Mathworks, 2014a, Natick,
153 Massachussets, USA). The resulting segmentation was used on the stiffness image to calculate
154 mean values of stiffness and standard deviation in each segmented region. Thus global
155 measurements obtain with the ROIs (Qbox™) are different from the global ones obtained with
156 segmentation as they do not represent the same surface.

157

158 **Histological analysis**

159 The impact of CA4P treatment 12 days after tumor graft was evaluated by histology. At
160 each time point of kinetics (day 13, 14 and 15) 3 mice of each group (control and treated) were
161 sacrificed, tumor was removed and quickly frozen in liquid nitrogen. Ten-micron frozen tissue
162 sections were placed on Polysine slides (Polysine®, CML, Nemours, France) and conserved in
163 -80°C before analysis. For each tumor, histological analysis was obtained in the central slice of
164 the tumor. In this slice, ten representative images were undertaken and analyzed. All stains were

165 performed at the same time and image digitization was made under identical lighting conditions
166 to insure that analyses from different slides were comparable.

167 Microvascular density (MVD) immunostaining for the evaluation of the vascularization
168 was performed using a CD31 (PECAM1) labeling consisting in a three-step procedure as
169 previously described (Youk et al., 2013; Seguin et al., 2013; Nico et al., 2008). The
170 quantification of the tumor vascularization was done using ImageJ software (version 1.50h)
171 (Abramoff 2004). The result was expressed in number of vessels per square millimeter (mm^2).
172 This parameter was referred to as MVD (microvascular density) in this paper. In order to
173 improve the quantification, a specific macro under ImageJ software was built to segment images
174 (appendix).

175 For necrosis assessment the haematoxylin eosin counterstain was performed (Robl et
176 al., 2016). The slides were washed in distilled water and fixed 20 min with paraformaldehyde
177 4% in phosphate buffer. The slides were then cleaned out with running tap water for 5 minutes
178 and stained in haematoxylin solution for 1 minute. A differentiation was made in saturated
179 solution of lithium for 5 minutes. Then, the slides were stained with eosin for 30 seconds.
180 Finally, they were dehydrated in alcohol and mounted with Eukitt® (Eukitt® quick-hardening
181 mounting medium, Sigma-Aldrich Chemie S.a.r.l, Saint-Louis, Missouri, USA). Two different
182 analyses of the necrosis were performed, global assessment of necrosis and percentage of
183 necrosis in the local areas (center and edges). Firstly, global assessment of necrosis was
184 calculated from dividing the necrosis area (pink zone in the image (fig. 4A, 4B)) by the total
185 surface of the tumor. The necrosis area was obtained by drawing manually the eosin staining
186 zones on images acquired at x25 magnification. Secondly, in order to get a value of necrosis in
187 the edge and in the center of the tumor, a quantification of the number of pink staining area per
188 square millimeter (mm^2) were done in 5 hot spots. These 5 hot spots were images at x200
189 magnification selected in 5 discrete locations within one slide per animal (appendix 2).

190 For fibrosis assessment: collagen fibers were stained with a Masson's trichromic
191 coloration staining kit (Masson's Trichrome Stain Kit 25088-1, Polysciences Europe GmbH,
192 Eppelheim, Germany) with some modifications adapted to immune-histochemical frozen
193 samples as described below. The slides were washed in distilled water and fixed during one
194 hour with paraformaldehyde 4% in a phosphate buffer. These slides were re-fixed in Bouin's
195 solution for 5 min at 56°C followed by a supplementary incubating of 15 min at room
196 temperature in order to improve the staining quality. The slides were washed with running tap
197 water for 5 minutes to remove the yellow color, and stained with Weigert's iron haematoxylin
198 working solution for 10 minutes and with Biebrich scarlet-acid fuchsin solution for 5 minutes.
199 The differentiation was made in phosphomolybdic-phosphotungstic acid solution for 10
200 minutes. The slides were then directly stained with aniline blue solution for 5 minutes, cleaned
201 out briefly in distilled water and differentiated in 1% acetic acid solution for 2-5 minutes. In
202 order to obtain the value of the fibrosis in the edges and the center of the tumor, a quantification
203 of the aniline blue staining was performed in 5 hot spot pictures in the center or in the edges of
204 the tumor.

205

206 **Statistical analysis**

207 Statistical Two-way ANOVA with Bonferroni post-tests were performed with Graphpad
208 software (Prism 5.01) to compare the control group and the treated group. Two-way ANOVA
209 determines how a response is affected by two factors: drug treatment and time. Bonferroni post-
210 tests were used to compare the control and the treated value at each time point. P values lower
211 than 0.05 ($P < 0.05$) were considered as statistically significantly different.

212 In the interest of compared different parameters in each condition, Spearman's rank
213 correlation has been applied, which makes no assumption about the distribution of the value,
214 but is based on ranks of the value.

215 At last the evolution of the relative difference in stiffness (RD_{stiff}) regarding the first day
216 of treatment was calculated by using the following formula:

$$217 \quad RD_{stiff} = \frac{(Eday_x - Eday_{12})}{Eday_{12}}, \quad \text{Eq. (1)}$$

218 where $Eday_x$ is the mean shear modulus in kPa at day x . This approach allowed us to quantify
219 the evolution of stiffness on the global population independently for each mouse.

220

221 **Results**

222 **Shear wave elastography**

223 On figure 1 is presented a shear modulus map (Fig. 1A) and a classical ultrasound image
224 (Fig. 1B) for one mouse. A ROI is placed on the shear modulus map covering the maximum
225 surface of the tumor. The mean stiffness value, maximum stiffness value, minimum stiffness
226 value and standard deviation values were extracted from each ROI (respectively on figure 1A:
227 5.1 kPa, 11.7 kPa, 1.0 kPa, 2.3 kPa). These values are averaged over multiple acquisition images
228 as described above providing the shear modulus mean values for each animal and day
229 (presented in figure 2B).

230

231 **Characterization of CT26 tumor stiffness as a function of time**

232 As shown in figure 2A, the tumor volume increased over time as previously observed
233 (Seguin et al., 2013). The shear modulus obtained by ultrasound imaging measurements also
234 increased with the tumor growth (Figure 2B).

235 A positive correlation ($r=0.59$, $p<0.0001$) was obtained between tumor volume and the
236 shear modulus parameter.

237

238 **Effect of Combretastatin A4P treatment**

239 Following the correlation between growth and stiffness, the effect of combretastatin on
240 tumor growth and elasticity changes was evaluated by shear wave elastography. Measurements
241 of tumor volume and elasticity post-combretastatin treatment were performed (Figure 3A and
242 3B). After CA4P treatment tumor volume did not increase from day 13 to day 15, while a
243 significant growth was noticed in the untreated control group. No change was measured in terms
244 of stiffness for both the treated and untreated groups. These results showed no correlation
245 between stiffness and volume for both groups ($r < 0.01$ control, $r = 0.05$ CA4P), in opposite to
246 what was observed prior day 12.

247 On figure 4A and 4B are presented histological slices of representative control tumor
248 and treated tumor respectively. An increase of the global necrotic area in the tumor between
249 control group and CA4P group was detected (Figure 4C, for example from 20.8 ± 3.3 % to
250 52.6 ± 3.0 % at day 13). On figures 4D and 4E are presented microscopic histological analyses
251 (200x), of the CD31 labeling in control and treated tumors respectively. The effect on vascular
252 density was found to be statistically different between the second and the third day of the
253 treatment (Figure 4F), in accordance to what was previously found (Tozer et al., 1999).

254

255 **Data refinement**

256 Histological analysis of data revealed significant changes, thus a refinement of the
257 stiffness analysis using normalization and segmentation of stiffness maps was made. After
258 normalization of the relative difference of stiffness as a function of the first day of treatment
259 (day 12) for each mouse, it is possible to observe a difference in the stiffness evolution between
260 both groups (Table 1). The stiffness values in the control group remained higher than the treated
261 group.

262 The value of shear modulus was obtained for the complete tumor area, and the effect of
263 the combretastatin is known to preferentially impact the center and leave a viable rim in

264 periphery of the tumor. Therefore, refinement of the analysis and evaluation of various
265 segmentations of the SWE images was applied. Figure 5 shows the stiffness map (A) and the
266 ultrasound B mode image (B) for a tissue sample were the segmentation of the edges and the
267 center was depicted on C (blue drawing) for one tumor.

268 The stiffness values were compared between global, center and edges of the tumor as a
269 function of time (Fig. 5 D, E, F). Regarding the global stiffness of the control mice, stiffness
270 changes significantly only after 15 days post implantation and 3 doses of combretastatin A4P.
271 This difference is clearer by looking specifically at the edges values (Fig. 5E) after map
272 segmentation.

273 Histological data for fibrosis and necrosis measurements are presented in Tables 2 and
274 3, respectively. Results were obtained on 5 random images in the center the tumor and on 5
275 random images in the edges of the tumor. These images were acquired in the histological slices
276 in order to correspond to the imaging plane. On Table 2, it can be appreciated that the percentage
277 of necrosis in the treated group increased as compared to the control group particularly in the
278 center of the tumor. On the other hand Table 3 shows that the fibrosis score is negligible in the
279 center and smaller than 2.5% on the edges in the control group. Meanwhile fibrosis increases
280 as a function of time in the treated group within the whole tumor and more drastically in the
281 edges of the tumor.

282

283 **Discussion**

284 In this study, CT26 tumors were analyzed using SWE and histological techniques in
285 order to assess stiffness, fibrosis, necrosis and vascularization, as a function of time.

286 Regarding stiffness, values were taken directly from the Aixplorer device which
287 assumes that medium are isotropic and purely elastic. However potential guided shear wave
288 can propagate in small layers, but this was not investigated. In fact guided shear wave can lead

289 to a lower estimate of the shear wave speed (Nguyen et al., 2011; Brum et al., 2014) but this
290 assumption was not taken into account in the present study.

291 As previously reported in the literature, stiffness increases with growing tumors (Seguin
292 et al., 2013). This was also observed in allografted breast cancer tumors (HBCx-3) by
293 Chamming's et al. (2013). A good correlation ($r= 0.59$) was found between volume assessment
294 and stiffness with a Spearman's rank correlation analysis. This result is consistent with the study
295 from Viana et al. who found that histological sections of CT26 tumor showed dense mass of
296 tumor cells with poor fibrovascular tissue (Viana et al., 2013).

297 Regarding only stiffness evolution as a function of time, multiple studies were
298 performed to better understand tumor behavior with treatment. In a clinical study, Athanasiou
299 et al. showed that stiffness assessment could help to monitor chemotherapy (Athanasiou et al.,
300 2015). However, this study was performed in a limited number of patients ($n=10$). In a
301 preclinical study by Juge et al, they observed using Magnetic Resonance Imaging, that the
302 tumor stroma in CT26 tumor represent only 10% of tumor area (Juge et al., 2012). In the same
303 work it was shown that the percent of proliferative cells increased from day 5 ($28.88 \pm 2.35\%$)
304 to day 11 ($39.59 \pm 3.92\%$). The cellularity increased significantly between day 5 (4775 ± 127
305 mm^2) and day 11 ($5917 \pm 398 \text{ mm}^2$; $P = .03$). The microvascular density also increased
306 significantly from day 5 ($224 \pm 3 \text{ mm}^2$) to day 11 ($299 \pm 37 \text{ mm}^2$, $P < .01$). Therefore, the
307 increased stiffness obtained by SWE from day 5 to day 12 in the present study is consistent
308 with the modification of cellular and microvascular density previously reported in the literature.
309 The aim of this study was to better understand histological and stiffness changes over a longer
310 treatment period consisting of repeated administration of the antivascular drug (CA4P).

311 In figures 4A, 4B, a **significant** necrotic area was found in the center of the treated
312 tumors (pink histological sample) following three days repeated injection of CA4P. This was
313 also reported in the literature (Ganau et al., 2014; Juge et al., 2012). The corresponding data

314 quantification presented in figure 4C was about 50 % and 20 % for treated and control tumors
315 respectively. Moreover, regarding MVD, the control group slightly increase as a function of
316 time while the treated group remained constant (fig. 4F). This was consistent with a lack of
317 vascularization resulting in some hypoxic areas which in turn led to necrosis. The level of
318 necrosis impacts the correlation between the tumor volume and the shear modulus for both the
319 control group and the treated group (Fig. 3A). Therefore, a poor correlation between stiffness
320 and volume was found for both groups ($r=0.00$ control, $r = 0.05$ CA4P) between days 13 and
321 15.

322 Changes in stiffness and relation with histological results are not so straightforward to
323 explain as tumor heterogeneity, in particular necrosis, plays a major role (Viana et al., 2013).
324 Using manual segmentation of the stiffness maps (Figure 5C), instead of a circular ROI
325 available on the Aixplorer device at the time of the study, allowed refining more precisely the
326 data leading to significant differences between control and treated groups at day 15. By using
327 this refinement, the stiffness values did not vary significantly in the center for both groups but
328 changed significantly in the edges. Interestingly, a significant difference in stiffness was found
329 between the control group and the treated group at days 14 and 15. This is consistent with the
330 well-known formation of a viable rim described after CA4P treatment (Tozer et al., 1999). To
331 go into further details, histological parameters and stiffness was simultaneously investigated.
332 Changes in stiffness were reflected by histological data (Tables 2&3). A strong necrosis both
333 in the center and in the edges was observed only for the treated group. A variation in fibrosis
334 was also evidenced for the treated group, although, this discrepancy was less important than
335 necrosis. Therefore for repeated administration of CA4P necrosis was the main factor
336 explaining the stiffness differences.

337

338 **Conclusion**

339 In this study, stiffness was found to be slightly lower after CA4P treatment and could
340 be related to modification in tissue organization like necrosis and fibrosis. The stiffness could
341 directly highlight changes in the growing tumor between days 5 and 12 corresponding to more
342 homogeneous tissue. After day 13, tumors are more heterogeneous due to the formation of
343 various areas (hypoxic zone, fibrosis, and necrosis) which require refining the analysis of the
344 SWE images to be able to detect stiffness discrepancies.

345

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354

355 **Appendix 1**

356 The ImageJ script used was a homemade macro dedicated to segment histological
357 parameter mainly based on contrast and color. The script was the following:

```
358 run("Enhance Contrast", "saturated=4 equalize"); run("Subtract Background...", "rolling=100  
359 light"); run("Despeckle"); run("Color Threshold..."); title = "binarization of vessels"; msg = "If  
360 necessary, use the \"Threshold\" tool to adjust the threshold, then click \"OK\".";  
361 waitForUser(title, msg); run("8-bit"); setMinAndMax(69, 255); run("Make Binary");  
362 run("Outline"); run("Analyze Particles...", "size=20-30000 circularity=0.00-1.00  
363 show=Outlines summarize");
```

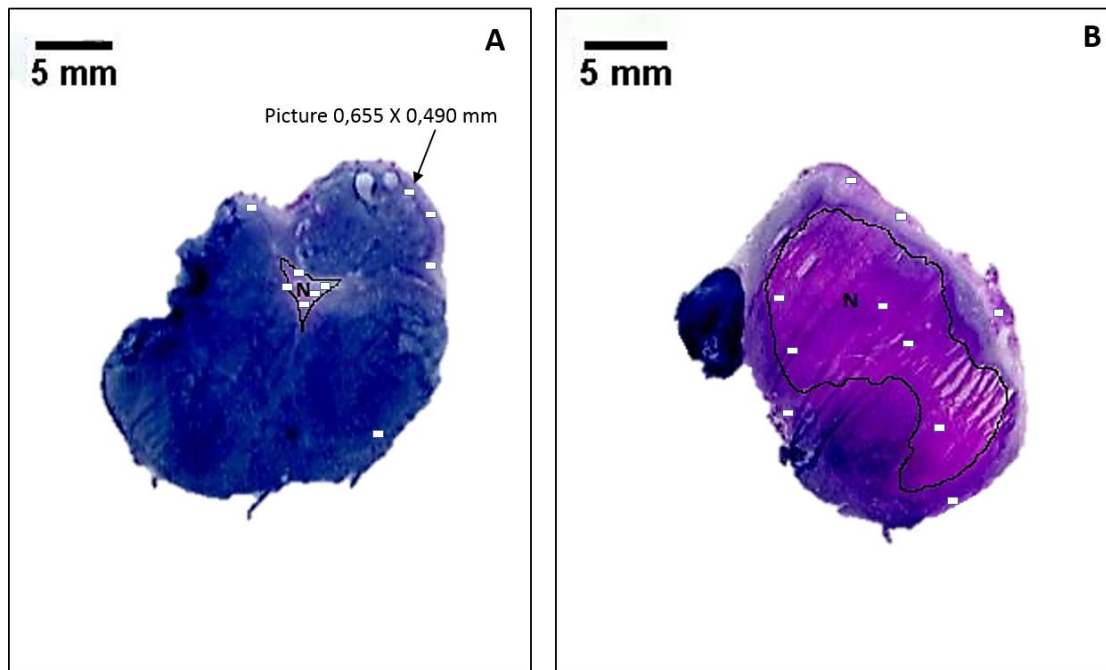

364 Threshold Color parameter:

365 Hue 41-233 pass untick, Saturation 84-255 pass tick, Brightness (automatic) pass tick,

366 Thresholding method : Default, Threshold color B&W, Color space HSB

367 **Appendix 2**

368 Histological method used for necrosis assessment:



369

370 Percentage of global necrosis: this percentage was calculated from dividing the necrosis area
371 (pink zone in the image (noted N)) by the total surface of the tumor. The necrosis area was
372 obtained by drawing manually the eosin staining zones on images acquired at x25
373 magnification.

374 Percentage of necrosis in the local areas: an evaluation of necrosis in the center and in the edges
375 was made in 5 hot spots. These 5 hot spots, were placed in the pink area located on the edge
376 and the center. An example of the positioning of theses hot spots was represented here (white
377 square), images was made at x200 magnification and have consequently a size of 0.655 x 0.490
378 mm.

379

380

381

382 **Conflict of interest statement**

383 M.T. is cofounder and shareholder of Supersonic Imagine Company. J.L.G. is scientific

384 consultant for Supersonic Imagine.

385

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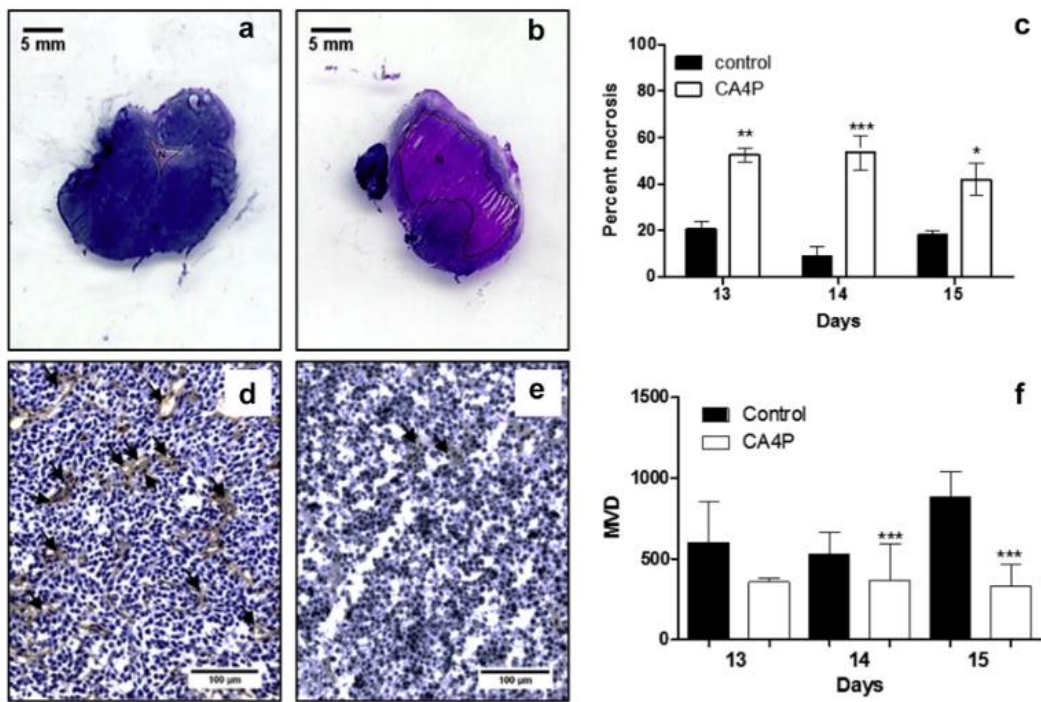
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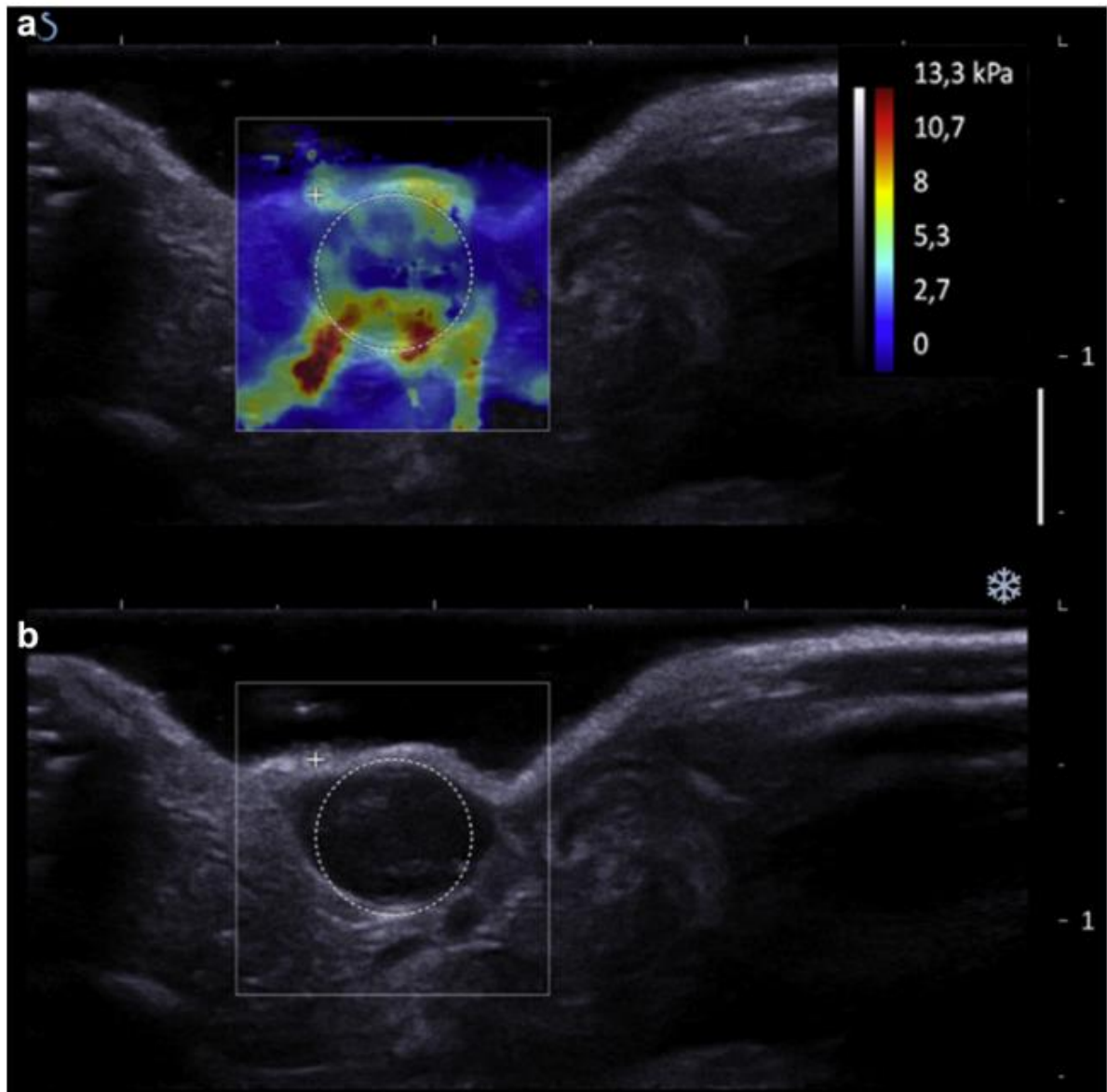
496 **Figure caption list**

497 **Figure 1:** A) Shear modulus map in the central sagittal plane. Mean value within the ROI (5
 498 mm diameter) is 5.1 ± 2.3 kPa, maximum: 11.7 kPa and minimum: 1.0 kPa. B) Corresponding
 499 ultrasound image in the central sagittal plane.



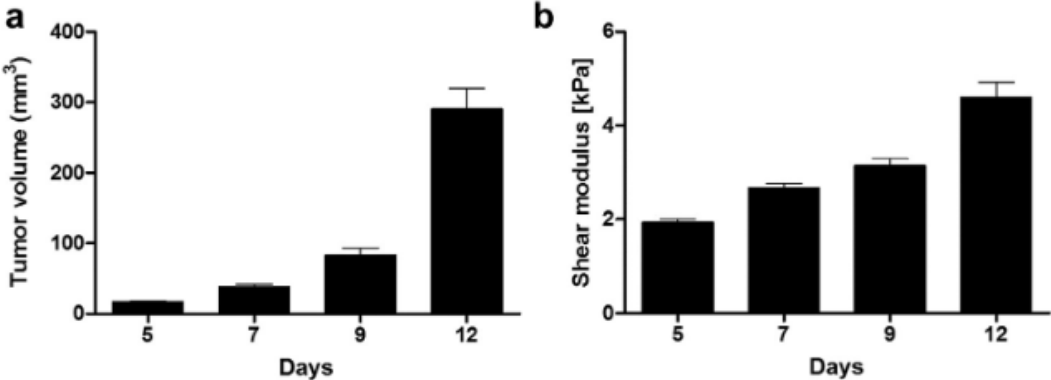
500

501 **Figure 2:** Longitudinal assessment of volume (A) and stiffness (B) from D5 to D12 post
 502 implantation of ectopic CT26 tumor obtained by ultrasound imaging and shear wave
 503 elastography. (Mean \pm SEM n=52).

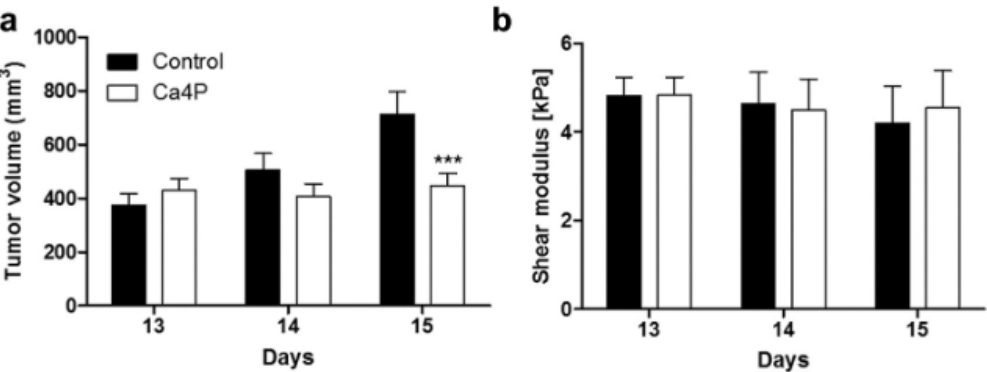


504

505 **Figure 3:** Longitudinal assessment of volume (A) and stiffness (B) of mice from D13 to D15
506 post implantation of ectopic CT26 tumor with and without CA4P treatment. Two-way ANOVA
507 with Bonferroni posttests *** $P < 0.001$. Values were obtained from Qbox measurements on the
508 Aixplorer device.

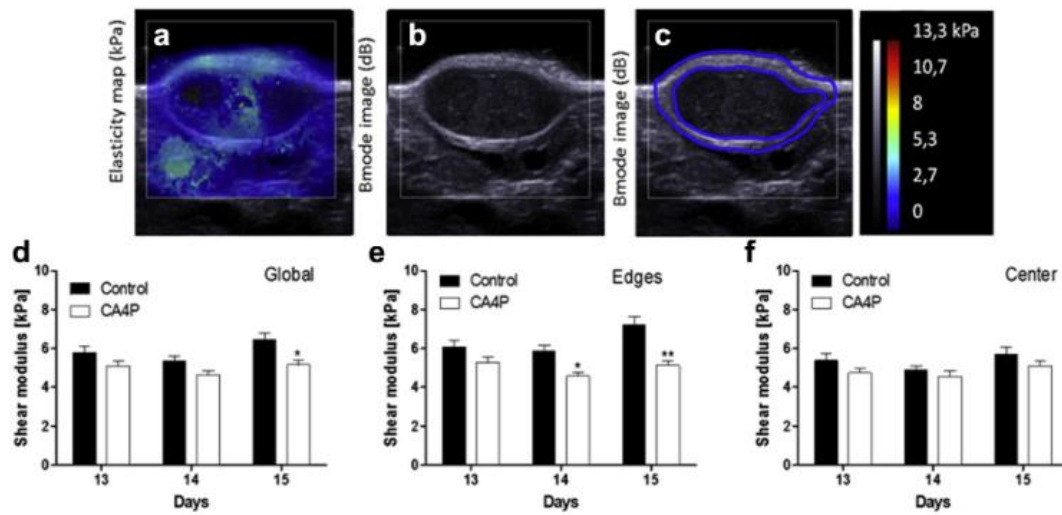


509
 510 **Figure 4:** Histological images of the CT26 tumor at day 15 before (A) and after treatment (B)
 511 with CA4P and corresponding data of necrosis percent (C, n= 3, mean ±SEM). CD31
 512 immunostaining of CT26 control (D) and treated (E) section 15 days after tumor implantation
 513 and corresponding data of MVD quantification (F, n= 3, mean ±SEM). Two-way ANOVA with
 514 Bonferroni posttests * P < 0.05, ** P<0.01, *** P<0.001.



515
 516 **Figure 5:** Procedure made for segmentation. Figure depicted in A tumor stiffness B the
 517 corresponding image in B-mode and in C the drawing area chosen for edges and center area
 518 (Blue). The corresponding data issue of the segmentation has presented as a function of time
 519 with or without CA4P treatment for the global tumor (D), the edges of the tumor (E) and the

520 center of the tumor (F).



521

522

523 **Table 1:** Relative difference in stiffness (RD_{stiff}) normalized at day 12 (dimensionless) for each

524 mice according to equation 1. Two-way ANOVA with Bonferroni posttests, *ns* not significant.

525 Values were obtained from Qbox measurements on the Aixplorer device.

526

Days	Control		CA4P		Two-way ANOVA		
	Mean \pm SEM	n	Mean \pm SEM	n	Difference	"P value"	Summary
13	0.295 \pm 0.071	26	0.041 \pm 0.098	26	-0,254	"P > 0.05"	ns
14	0.198 \pm 0.124	14	0.007 \pm 0.126	22	-0,191	"P > 0.05"	ns
15	0.013 \pm 0.094	9	0.059 \pm 0.140	19	0,045	"P > 0.05"	ns

527

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530

531 **Table 2:** Necrosis area expressed in percentage hot spot (Mean \pm SEM (n=3)) Two-way ANOVA with
 532 Bonferroni posttests, ns not significant, ** P < 0.01, *** P<0.001

533

534

Days	Center (%)		Edges (%)	
	Control	CA4P	Control	CA4P
13	50.69 \pm 10.87	97.08 \pm 1.42, ***	34.63 \pm 6.48	77.23 \pm 18.43, ns
14	33.65 \pm 1.47	98.97 \pm 1.03, ***	9.86 \pm 4.97	87.24 \pm 12.34, **
15	54.53 \pm 8.90	97.46 \pm 2.54, ***	44.79 \pm 13.94	80.23 \pm 13.41, ns

535

536

537 **Table 3:** Fibrosis area expressed in percentage hot spot (Mean \pm SEM (n=3)) Two-way ANOVA with
 538 Bonferroni posttests, ns not significant.

539

540

Days	Center (%)		Edges (%)	
	Control	CA4P	Control	CA4P
13	0.00 \pm 0.00	0.00 \pm 0.00 ns	0.69 \pm 0.25	2.31 \pm 0.75 ns
14	0.02 \pm 0.02	0.59 \pm 0.31 ns	1.96 \pm 0.60	4.34 \pm 2.08 ns
15	0.01 \pm 0.01	0.69 \pm 0.40 ns	2.27 \pm 1.29	5.03 \pm 0.98 ns

541