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1 **Investigating the Contribution of Collagen to the Tumor Biomechanical**  
2 **Phenotype with Non-invasive Magnetic Resonance Elastography**

3  
4  
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40  
41  
42 **Research Article**

43  
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45  
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1 **Abstract (241 words)**

2 Increased stiffness in the extracellular matrix (ECM) contributes to tumor progression and  
3 metastasis. Therefore, stromal modulating therapies and accompanying biomarkers are  
4 being developed to target ECM stiffness. Magnetic resonance (MR) elastography can  
5 noninvasively and quantitatively map the viscoelastic properties of tumors *in vivo* and thus  
6 has clear clinical applications. Herein, we used MR elastography, coupled with  
7 computational histopathology, to interrogate the contribution of collagen to tumor  
8 biomechanical phenotype and evaluate its sensitivity to collagenase-induced stromal  
9 modulation. Elasticity ( $G_d$ ) and viscosity ( $G_l$ ) were significantly greater for orthotopic BT-474  
10 ( $G_d=5.9\pm 0.2\text{kPa}$ ,  $G_l=4.7\pm 0.2\text{kPa}$ ,  $n=7$ ) and luc-MDA-MB-231-LM2-4 ( $G_d=7.9\pm 0.4\text{kPa}$ ,  
11  $G_l=6.0\pm 0.2\text{kPa}$ ,  $n=6$ ) breast cancer xenografts, and luc-PANC1 ( $G_d=6.9\pm 0.3\text{kPa}$ ,  
12  $G_l=6.2\pm 0.2\text{kPa}$ ,  $n=7$ ) pancreatic cancer xenografts, compared to tumors associated with the  
13 nervous system, including GTML/*Trp53<sup>K1/K1</sup>* medulloblastoma ( $G_d=3.5\pm 0.2\text{kPa}$ ,  
14  $G_l=2.3\pm 0.2\text{kPa}$ ,  $n=7$ ), orthotopic luc-D-212-MG ( $G_d=3.5\pm 0.2\text{kPa}$ ,  $G_l=2.3\pm 0.2\text{kPa}$ ,  $n=7$ ), luc-  
15 RG2 ( $G_d=3.5\pm 0.2\text{kPa}$ ,  $G_l=2.3\pm 0.2\text{kPa}$   $n=5$ ) and luc-U-87-MG ( $G_d=3.5\pm 0.2\text{kPa}$ ,  
16  $G_l=2.3\pm 0.2\text{kPa}$   $n=8$ ) glioblastoma xenografts, intracranially propagated luc-MDA-MB-231-  
17 LM2-4 ( $G_d=3.7\pm 0.2\text{kPa}$ ,  $G_l=2.2\pm 0.1\text{kPa}$ ,  $n=7$ ) breast cancer xenografts, and Th-*MYCN*  
18 neuroblastomas ( $G_d=3.5\pm 0.2\text{kPa}$ ,  $G_l=2.3\pm 0.2\text{kPa}$ ,  $n=5$ ). Positive correlations between both  
19 elasticity ( $r=0.72$ ,  $p<0.0001$ ) and viscosity ( $r=0.78$ ,  $p<0.0001$ ) were determined with collagen  
20 fraction, but not with cellular or vascular density. Treatment with collagenase significantly  
21 reduced  $G_d$  ( $p=0.002$ ) and  $G_l$  ( $p=0.0006$ ) in orthotopic breast tumors. Texture analysis of  
22 extracted images of picosirius red staining revealed significant negative correlations of  
23 entropy with  $G_d$  ( $r=-0.69$ ,  $p<0.0001$ ) and  $G_l$  ( $r=-0.76$ ,  $p<0.0001$ ), and positive correlations of  
24 fractal dimension with  $G_d$  ( $r=0.75$ ,  $p<0.0001$ ) and  $G_l$  ( $r=0.78$ ,  $p<0.0001$ ). MR elastography  
25 can thus provide sensitive imaging biomarkers of tumor collagen deposition and its  
26 therapeutic modulation.

27

28 **Significance (17 words)**

29

30 MR elastography enables non-invasive detection of tumor stiffness and will aid in the  
31 development of ECM-targeting therapies.

32

33

34

## 1 Introduction

2

3 Aberrant tensional homeostasis and increased stiffness are hallmarks of cancer. The origin  
4 of elevated tumor stiffness is not fully understood, but may often reflect increased  
5 mechanical stress associated with rapid tissue expansion and compressed vasculature and  
6 lymphatics, and extracellular matrix (ECM) rigidity. There is much evidence showing that  
7 increased tissue stiffness contributes to malignant transformation, tumor progression and  
8 metastasis (1,2). The elevated solid stress and interstitial fluid pressure (IFP) that may drive  
9 increased tumor stiffness are also two major obstacles to efficient tumor drug delivery (3).

10

11 Both breast and pancreatic cancers are characterized by excessive desmoplastic stromal  
12 reaction and dense ECM (4,5). Collagen, the principal component of the fibrillary protein  
13 network within the ECM, is the major contributor to ECM stiffening, is strongly implicated in  
14 tumor evolution and progression, and is associated with poor patient prognosis (6-9).  
15 Increased collagen deposition and enhanced matrix cross-linking occurs with progressive  
16 structural remodeling of the ECM scaffold which facilitates tumor growth (9). Suppression of  
17 collagen synthesis inhibits tumor growth and metastasis, and enhances drug penetration  
18 (10).

19

20 Significant efforts are currently focused on targeting tumor ECM stiffness for therapeutic gain  
21 (2,4,11-13). The development of stromal modulating therapies would benefit from non-  
22 invasive imaging biomarkers to inform on changes associated with therapeutic efficacy. A  
23 number of innovative magnetic resonance (MR) and ultrasound (US) imaging techniques are  
24 being exploited to image the viscoelastic and other mechanical properties of tissue *in vivo*  
25 (14-17). One approach, MR elastography, is being used to visualize and measure tissue  
26 elasticity and viscosity *in vivo*. MR elastography yields quantitative images, and therefore  
27 imaging biomarkers, that map the absolute value of the complex shear modulus  $G^*$  in terms  
28 of its two components, the elasticity modulus  $G_d$  (a measure of the ability of an object to  
29 resume a normal shape after being stretched or compressed) and the viscosity modulus  $G_v$   
30 (a measure of resistance to gradual deformation by shear or tensile stress). We and others  
31 have demonstrated, in pre-clinical tumor models and cancer patients *in vivo*, the potential of  
32 the MR elastography-derived mechanical phenotype to inform on the underlying tumor  
33 microstructure and treatment-induced changes to its integrity (18-25).

34

35 Early imaging biomarker development demands close imaging-pathology correlation to  
36 understand the biological processes underpinning the imaging measurement, which can be  
37 meaningfully studied using animal models (26-28). The systematic evaluation of tumor

1 stromal components and their contribution to tissue stiffness as measured by MR  
2 elastography is in its infancy. This study describes the use of MR elastography, coupled with  
3 computational histopathology, to interrogate the contribution of the collagen network to the  
4 biomechanical phenotype imaged *in vivo* in a wide range of orthotopically-propagated and  
5 spontaneously arising transgenic models with disparate pathologies. The sensitivity of MR  
6 elastography to monitor stromal modulation *in vivo* following administration of collagenase is  
7 also demonstrated.

8

9

## 1 **Materials and Methods**

2

### 3 **Cells**

4 All cell lines used in this study tested negative for mycoplasma infection at the time of tumor  
5 implantation and human cells were authenticated by short tandem repeat (STR) profiling.  
6 Their origins, provenance and culture conditions are summarized in Supplementary Table 1  
7 and 2.

8

### 9 **Animals & Tumor Models**

10 All animal experiments were approved by The Institute of Cancer Research Animal Welfare  
11 and Ethical Review Body, and performed in accordance with the UK Home Office Animals  
12 (Scientific Procedures) Act 1986, the United Kingdom National Cancer Research Institute  
13 guidelines for the welfare of animals in cancer research (29), and reported according to the  
14 ARRIVE (animal research: reporting *in vivo* experiments) guidelines (30). Mice were housed  
15 in specific pathogen-free rooms in autoclaved, aseptic microisolator cages with a maximum  
16 of 5 animals per cage. Mice were allowed access to sterile food and water *ad libitum*. A total  
17 of 84 mice were enrolled and a summary of the *in vivo* models used in this study is  
18 presented in Table 1.

19

#### 20 *Intracranial tumor propagation*

21 Human luc-U-87 MG glioblastoma ( $5 \times 10^4$ ), rat luc-RG2 glioma ( $5 \times 10^3$ ), human luc-D-212  
22 MG pediatric hemispheric giant-cell glioblastoma ( $5 \times 10^3$ ), or human metastatic luc-MDA-  
23 MB-231 LM2-4 breast cancer ( $5 \times 10^3$ ) cells were implanted supratentorially in the brains of  
24 adult female athymic NCr-Foxn1<sup>nu</sup> mice (Charles River, Margate, UK) as previously  
25 described (19). Animals were anesthetized using 1-2% isoflurane in oxygen (1 l/min). A ~1  
26 cm incision was made in the skin on the top of the head, and a 1mm hole drilled using a  
27 surgical bone microdrill (Harvard Apparatus, Edenbridge, UK). Cell suspension (5  $\mu$ l) was  
28 then injected at a depth of 3 mm from the dura, at a rate of 2  $\mu$ l/min, using a 10  $\mu$ l syringe  
29 (VWR International, Lutterworth, UK) and a nanomite syringe pump (Harvard Apparatus).  
30 The needle was removed 3 minutes after completion of the injection and the skin repaired  
31 with Vetbond™ Tissue Adhesive (3M Animal Care Products, St Paul, MN, USA).

32

33 Tumor establishment and growth were monitored with bioluminescence imaging (BLI) using  
34 a Xenogen IVIS® 200 system coupled with LivingImage software (Caliper Life Sciences,  
35 Runcorn, UK). Luciferin (150mg/kg, Caliper Life Sciences) was administered  
36 intraperitoneally 10 minutes before imaging. MR elastography was performed when the BLI  
37 photon flux reached a threshold value previously determined to represent a tumor of

1 approximately 30-40 mm<sup>3</sup>, a volume considered of sufficient size to acquire MR  
2 elastography data but not large enough to cause neurological effects in the mice. The  
3 average time from implantation to imaging was 18 days for the luc-U-87 MG and luc-MDA-  
4 MB-231 LM2-4 tumors, 22 days for the luc-RG2 tumors and 45 days for the luc-D-212 MG  
5 glioblastomas.

6

#### 7 *GTML/Trp53<sup>KI/KI</sup> transgenic model of medulloblastoma*

8 The generation of the GTML/Trp53<sup>KI/KI</sup> mice has been previously reported (31). Mice were  
9 genotyped to detect the presence of human *MYCN* and *Trp53* transgenes. Male and female  
10 mice were monitored twice weekly for the development of a BLI signal from the midbrain.  
11 MR elastography was performed when the photon flux reached a threshold value previously  
12 determined to represent a tumor of approximately 20-30 mm<sup>3</sup>.

13

#### 14 *Th-MYCN transgenic model of neuroblastoma*

15 Transgenic Th-MYCN mice were genotyped to detect the presence of the human *MYCN*  
16 transgene (27). Both male and female hemizygous mice were used, which spontaneously  
17 developed palpable abdominal tumors between 50–130 days with a 25% penetrance. Tumor  
18 progression was monitored weekly by palpation by an experienced technician until the tumor  
19 reached a diameter greater than ~5 mm, at which point they underwent MR elastography.

20

#### 21 *Subcutaneous U87-MG xenografts*

22 Adult female NCr-*Foxn1<sup>nu</sup>* mice were injected subcutaneously in the flank with 2 x 10<sup>6</sup> luc-U-  
23 87-MG cells. Tumor development was monitored weekly by caliper measurements, and MR  
24 elastography performed when tumors reached a diameter of ~7 mm.

25

#### 26 *Orthotopic models of breast and pancreatic cancer*

27 Human luc-MDA-MB-231 LM2-4 or BT-474 breast cancer cells (5 x 10<sup>6</sup>) were injected into  
28 the third abdominal fat pad of adult female NCr-*Foxn1<sup>nu</sup>* mice (100 µl cell suspension in PBS  
29 and matrigel (1:1)). A 17β-estradiol pellet (60-day release, Innovative Research of America,  
30 Sarasota, FL, USA) was implanted in the neck nape one day before implantation of BT-474  
31 cells. Tumor development was monitored weekly by caliper measurements, and MR  
32 elastography performed when tumors reached a diameter of ~7 mm.

33

34 For the orthotopic propagation of pancreatic cancer xenografts, a small incision was made  
35 on the left flank of adult female athymic CD1-*Foxn1<sup>nu</sup>* mice (Charles River, Margate, UK)  
36 through the skin and peritoneum, the pancreas exteriorized, and human luc-Panc-1 (1 x  
37 10<sup>7</sup> cells in suspension in PBS and matrigel (1:1)) injected using a Hamilton syringe. The



1 pancreas was then returned into the abdominal cavity and the incision sutured. Successful  
2 engraftment and tumor progression were confirmed using BLI, and MR elastography  
3 performed ~90 days post-implantation.

4

### 5 **Response to Collagenase**

6 Orthotopic parental MDA-MB-231 and BT-474 breast cancer xenografts were propagated as  
7 described above. MR elastography was performed on established tumors 24 hours prior to  
8 and 5 hours after intravenous treatment with either collagenase (62U/kg, bacterial  
9 collagenase from *Clostridium histolyticum*, Sigma-Aldrich, UK) or saline.

10

### 11 **MRI and MR elastography data acquisition and analysis**

12 MRI was performed on a 7T horizontal bore MicroImaging system (Bruker, Ettlingen,  
13 Germany) using a 3cm birdcage volume coil. Tumor-bearing mice were anesthetized with a  
14 10ml/kg intraperitoneal injection of fentanyl citrate (0.315mg/ml) plus fluanisone (10mg/ml  
15 (Hypnorm; Janssen Pharmaceutical Ltd., High Wycombe, UK)), midazolam (5mg/ml  
16 (Hypnovel; Roche)), and sterile water (used at a ratio of 1:1:2). The mouse core temperature  
17 was maintained at 37°C with warm air blown through the magnet bore.

18

19 Anatomical T<sub>2</sub>-weighted images (using a rapid acquisition with refocused echoes (RARE)  
20 sequence, with TE = 36ms, TR = 4.5s, RARE factor = 8, 40 contiguous 1mm thick  
21 transverse slices, 1 average, matrix size 128×128 over a 3×3cm field of view (FOV)) were  
22 used to localize and determine the tumor volume, plan the MR elastography acquisition, and  
23 optimize the local field homogeneity over the region of interest (ROI) using the FASTMAP  
24 algorithm.

25

26 MR elastography was performed as previously described (18,19). The mechanical  
27 vibrations, generated by an electromagnetic shaker (Brüel & Kjaer, Nærum, Denmark), were  
28 transmitted through a flexible nylon rod to either *i*) a square piston with a concave curved  
29 face positioned on the mouse head for intracranial tumors, or directly on the skin over  
30 subcutaneous luc-U87-MG tumors and orthotopically propagated breast cancer xenografts,  
31 or *ii*) a round flat-faced piston placed on the abdomen above a palpated tumor (Th-*MYCN* or  
32 orthotopic pancreatic tumor), all positioned within the volume coil at the isocenter of the  
33 magnetic field. MR elastography was performed using mechanical excitations at a vibration  
34 frequency of 1000 Hz, exciting the shaker with a voltage that generated mechanical waves  
35 inside the tumor with amplitude greater than 0.5 μm. A 2D spin-echo sequence incorporating  
36 sinusoidal motion-sensitizing gradients synchronized to the mechanical excitation was used.  
37 Data were acquired in 3 orthogonal directions, from 10 contiguous transverse slices (300 μm

1 thick), using 2 averages of 64 phase encoding steps over a 1.92×1.92 cm FOV, with TE = 27  
2 ms, TR = 1001 ms and 8 time sampling steps, giving an isotropic spatial sampling of  
3 300×300×300 μm of the mechanical wave propagation displacement inside the tumor. The  
4 total acquisition time was ~ 51 min. Finally, high resolution T<sub>2</sub>-weighted RARE images were  
5 acquired from the same ten contiguous transverse slices, (TE = 36 ms, TR = 4.5 s, RARE  
6 factor = 8, 300 μm thick, 10 averages, matrix size 128×128 over a 1.92×1.92cm FOV).

7

8 *Image Reconstruction and Analysis.* Parametric maps of the absolute value of the complex  
9 shear modulus  $|G^*|$ , elasticity  $G_d$  and viscosity  $G_l$  (where  $|G^*| = G_d + iG_l$ ) were reconstructed  
10 using in-house software from the three-dimensional displacement vector measured as  
11 described above, and using the following equation (32):

12 
$$-\rho\omega^2\vec{q} = G^*\nabla^2\vec{q}, \vec{q} = \vec{\nabla} \times \vec{u} \in C^3,$$

13 where  $\vec{q}$  is the complex-valued curl of the measured displacement field  $\vec{u}$ ,  $\rho$  is the density of  
14 the material and  $\omega$  is the angular frequency. For each slice,  $G_d$  and  $G_l$  (kPa) were  
15 determined pixelwise from a ROI covering the whole tumor delineated from the high  
16 resolution T<sub>2</sub>-weighted images.

17

### 18 **Computational histopathology**

19

20 *Tissue preparation:* Guided by the T<sub>2</sub>-weighted MR images, tumors were carefully excised  
21 and orientated for subsequent histopathological processing. Adjacent formalin-fixed paraffin  
22 embedded sections (3 μm) were cut and tinctorially stained with picosirius red (for collagen I  
23 and III), hematoxylin and eosin (H&E, for cellularity), or immunohistochemically processed  
24 for detection of the murine vascular endothelial marker CD31 (rabbit EP3095, Millipore,  
25 Watford, UK), using diaminobenzidine (DAB) as the chromogen.

26

27 *Digitized histology:* Whole-slide images were digitized using a NanoZoomer XR scanner  
28 (20x magnification, 0.46μm resolution, Hamamatsu, Japan). ROIs of viable tumor and  
29 necrosis for each sample were independently provided. Histology images were subsequently  
30 split into tiles of 2000×2000 pixels (jpeg).

31

32 *Picosirius red staining segmentation:* A macro was written in Fiji (<https://fiji.sc/>) to segment  
33 picosirius red staining from each tile using ImageJ/Fiji plugins (Java 8). Images were first  
34 converted from the RGB color space into the green-red (a\*) color channel of the CIELAB  
35 color space (lightness, green–red and blue–yellow), and subsequently thresholded to  
36 segment the picosirius red staining from the background. Following appraisal of automatic

1 and manual thresholding, two manual thresholds were chosen to achieve the optimal  
2 picrosirius red staining segmentation across all samples, whilst compensating for variations  
3 in staining intensity and complex background values associated with the different cancer  
4 pathologies. A threshold value of above +17 was used in the a\* color channel for the  
5 majority of tumor types, increased to +23 for medulloblastomas and neuroblastomas arising  
6 in the GTML/*Trp53*<sup>KI/KI</sup> and Th-*MYCN* transgenic mice respectively, and subcutaneous luc-U-  
7 87-MG tumors. The segmentation algorithm was tested using independent annotation, from  
8 a single observer blinded to the algorithm's result, of stained/non-stained points (3920/3478)  
9 on 17 different samples across all cancer types, giving an accuracy of 95% with 91%  
10 sensitivity.

11

12 *Cell segmentation from H&E-stained sections:* Images were processed using the EImage  
13 Bioconductor package (33). Cell nuclei were extracted from each image tile using the Otsu  
14 thresholding algorithm, followed by morphological opening to delete the noisy structures and  
15 the Watershed algorithm to separate clustered nuclei (34).

16

17 *CD31 segmentation from CD31-stained immunohistochemistry sections:* A macro was  
18 written in Fiji to extract DAB staining from each tile by applying color unmixing to extract the  
19 brown color channel, followed by application of the maximum entropy threshold detection  
20 method, both using ImageJ/Fiji plugins (Java 8) as previously described (27).

21

22 *Generation of collagen fraction, cellularity and vascular density parametric maps:* Whole-  
23 slide images of picrosirius red staining, segmented cells, and CD31 staining were converted  
24 into binary and processed to match MR elastography resolution (300x300 $\mu$ m), with the  
25 fraction of pixels occupied by the center of each cell nucleus, picrosirius red and CD31  
26 staining within 664x664 pixel-regions representing a single pixel in the final calculated maps.  
27 Quantitative analysis of each stain was performed from one histological tumor section  
28 aligned with the central slice of the elastogram. Necrotic areas visible on both T<sub>2</sub>-weighted  
29 images and corresponding histopathology slides were subtracted from the viable tumor by  
30 manual regional segmentation and excluded from the quantitative analysis.

31

32 *Texture analysis:* We evaluated the two-dimensional heterogeneity of collagen distribution by  
33 quantifying entropy and fractal dimension (FD), as described by Nieskoski et al (35), on the  
34 extracted collagen parametric maps. The texture analysis was implemented in Matlab  
35 (R2018b, Mathworks, Natick, MA, USA). Necrotic areas were subtracted from the viable  
36 tumor and excluded from the analysis. Entropy was quantified to measure the irregularity of  
37 collagen distribution by applying the "entropy" function of Matlab, which uses the equation:

1 
$$Entropy = - \sum_{i=1}^N p * \log_2 p$$

2 , where p represents the normalized histogram count of the collagen fraction. FD defines the  
3 complexity (textural roughness) of collagen distribution within the tumor samples. The  
4 Hausdorff (box-counting) method was applied in the histology images within the 664x664  
5 pixel-regions described earlier, using the equation:

6 
$$FD = \log(N(e))/\log\left(\frac{1}{e}\right)$$

7  
8 , where e represents the box size set to the size of the image and N(e) corresponds to the  
9 number of boxes of size 'e' which contain collagen. The median value of the generated FD  
10 maps (excluding necrotic areas) was used as the tumor's FD value.

11

12

### 13 **Statistical Analysis**

14 Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., La  
15 Jolla, USA). Unless stated otherwise, data are presented as mean  $\pm$  1 s.e.m. Significant  
16 differences in quantitative MR elastography parameters between tumor types, and in relative  
17 treatment-induced changes, were identified using the non-parametric Mann-Whitney U-test  
18 with a 5% level of significance, whilst significant changes in MR elastography parameters  
19 with treatment were identified using the Wilcoxon matched-pairs signed ranks test with a 5%  
20 level of significance. Significant correlations were determined using linear regression  
21 analysis with a 5% level of significance using the robust regression and outlier removal  
22 approach (36).

23

24

## 1 Results

2  
3 MR elastography was successfully performed in all mice, yielding an intertumoral coefficient  
4 of variation (CoV) of 13.0 and 15.4 % for repeated measurements of  $G_d$  and  $G_l$  respectively  
5 (Supplementary methods). MR elastography revealed a heterogeneous distribution of  
6 elasticity and viscosity across the nine orthotopic and transgenic models of cancer  
7 investigated (Figure 1A). Pronounced contrast between the established tumor and the  
8 surrounding brain was clearly evident in the intracranial models, with the lesion boundaries  
9 aligning with those seen in the high-resolution T<sub>2</sub>-weighted images. Quantitative analysis of  
10 the MR elastography data demonstrated a wide range in  $G_d$  and  $G_l$  values across the  
11 models, from  $3.5 \pm 0.2$  and  $2.2 \pm 0.2$  kPa respectively in the GTML/*Trp53*<sup>K1/K1</sup> transgenic  
12 mouse model of medulloblastoma, to  $7.9 \pm 0.4$  and  $6.0 \pm 0.2$  kPa in the orthotopic luc-MDA-  
13 MB-231 LM2-4 mammary carcinomas (Figure 1B). Collectively, elasticity and viscosity were  
14 significantly ( $p < 0.0001$ ) greater for the orthotopically-propagated breast and pancreas  
15 models, compared to the tumors associated with the central or peripheral nervous system.

16  
17 Intravenous injection of collagenase resulted in a clear overall reduction in the elasticity and  
18 viscosity of orthotopic MDA-MB-231 and BT-474 mammary tumors, as measured by MR  
19 elastography, 5 hours after administration (Figure 2A). Tumor regions exhibiting relatively  
20 high  $G_d$  and  $G_l$  pre-treatment were typically reduced following challenge with collagenase.  
21 No similar response was evident in the vehicle-treated mice. Collectively, collagenase  
22 resulted in a significant reduction in both  $G_d$  ( $6.0 \pm 0.4$  kPa to  $4.9 \pm 0.4$  kPa,  $p = 0.001$ ) and  $G_l$   
23 ( $3.8 \pm 0.6$  kPa to  $3.0 \pm 0.5$  kPa,  $p = 0.001$ ), which was not observed in the vehicle-treated  
24 cohort ( $G_d$ :  $5.1 \pm 0.4$  kPa to  $5.4 \pm 0.3$  kPa,  $p = 0.3$ ;  $G_l$ :  $3.0 \pm 0.6$  kPa to  $3.4 \pm 0.5$  kPa,  $p = 0.13$ ).  
25 Relative changes in both  $G_d$  and  $G_l$  were significantly different between the collagenase and  
26 vehicle-treated cohorts ( $p = 0.0008$  and  $p = 0.0004$ , Figure 2B).

27  
28 To investigate the pathological determinants of the regional variations in tumor viscoelasticity  
29 seen *in vivo*, parametric maps of  $G_d$  and  $G_l$  were compared with maps of picosirius red  
30 (collagen I & III), H&E (cellularity) and CD31 (vascular density) staining, automatically  
31 segmented from high-resolution images of aligned tissue sections from the same tumor  
32 (Figure 3A). In the the GTML/*Trp53*<sup>K1/K1</sup> transgenic mouse model, which exhibited the lowest  
33 mean  $G_d$  and  $G_l$ , of all the models investigated, tumors presented with a thin layer-like region  
34 of elevated  $G_d$  and  $G_l$  spatially associated with strong picosirius red staining adjacent to the  
35 skull, consistent with tumor invasion into the collagen-rich meninges (Figure 3B). In general,  
36 but especially in breast and pancreatic tumors, regions demonstrating high values of  $G_d$  and  
37  $G_l$  spatially corresponded to cellular regions with higher deposition of collagen. In some

1 tumors, histologically-defined regions of extensive tissue damage (e.g. necrosis) were  
2 spatially associated with areas of markedly lower elasticity and viscosity. It is important to  
3 note the relative softness of these necrotic regions irrespective of their also having high  
4 collagen content. These regions were excluded from the subsequent quantitative analysis.  
5 Quantitative analysis identified statistically significant positive inter-tumor correlations of both  
6 tumor-mean elasticity  $G_d$  ( $r=0.72$ ,  $p<0.0001$ ) and viscosity  $G_l$  ( $r=0.78$ ,  $p<0.0001$ ) with tumor-  
7 mean collagen fraction, but not with tumor-mean cellularity and vascular density (Figure 3C).

8  
9 The irregularity of collagen distribution and deposition, and its relationship to tumor  
10 viscoelasticity *in vivo*, was further investigated using texture analysis of the extracted images  
11 of picrosirius red staining. Significant negative correlations of entropy with  $G_d$  ( $r=-0.69$ ,  
12  $p<0.0001$ ) and  $G_l$  ( $r=-0.76$ ,  $p<0.0001$ ), and positive correlations of FD with  $G_d$  ( $r=0.75$ ,  
13  $p<0.0001$ ) and  $G_l$  ( $r=0.78$ ,  $p<0.0001$ ) were found (Figure 4A and B). Entropy values close to  
14 zero and relatively high values of FD determined in the BT-474, luc-PANC-1 and luc-MDA-  
15 MB-231 LM2-4 tumors are consistent with the presence of a homogeneous and dense  
16 collagen network. Note that in the models investigated, increasing collagen content was  
17 associated with both increasing density and uniformity of its distribution, as shown by the  
18 mono-exponential relationship of entropy ( $y= 3.86e^{-0.24x}$ ,  $r^2=0.76$ ) and logarithmic relationship  
19 of FD ( $y= 0.15\ln x + 1.16$ ,  $r^2=0.98$ ) with collagen fraction, respectively (Figure 4C).

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## 1 Discussion

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ECM stiffening is increasingly recognized as a major mechanical signal, which alters cell behavior and in part confers to cancer cell hallmark capabilities including sustained growth, invasion and metastasis (6,7,37-42). ECM stiffening is also associated with increased solid stress and IFP, two other hallmarks of tumor mechanobiology that induce blood and lymphatic vessel compression, and reduced transcapillary transport respectively, and which impair effective drug delivery (3). Disrupting the crosstalk between cancer cells and the ECM, as well as reversing ECM stiffness, solid stress or IFP, thus represents a promising therapeutic strategy (11). The clinical development of stromal modulating therapies would be facilitated and accelerated by non-invasive imaging methods to longitudinally image and quantify tumor mechanical properties *in vivo* (15-17).

In this pre-clinical study, our quantitative MR elastography tumor data, combined with aligned computational histopathology, showed that elevated tumor  $G_d$  and  $G_l$  correlated with increased collagen deposition across a wide range of clinically-relevant tumor models with disparate pathologies. Our data particularly highlight the relative softness of tumors arising in the nervous system, and the predicted elevated stiffness of orthotopic breast and pancreatic models. Furthermore, modulation of the collagen network with bacterial collagenase in two relatively stiff orthotopic models of breast cancer reveal a marked reduction in both elasticity and viscosity. Collectively our results demonstrate that the deposition and density of the collagen fiber network is a major determinant of the tumor biomechanical phenotype, for which MR elastography provides sensitive *in vivo* imaging biomarkers, including biomarkers of its pharmacological modulation.

Our MR elastography study also aligns with several established concepts in tissue mechanics. The elastic properties of tissues are defined by the mechanical characteristics of their cells and their surrounding ECM, yet tissue macroscopic viscoelastic properties are not greatly affected by differences in cell morphology and density, but are largely governed by their ECM composition (43). Herein, histologically-confirmed regions of tissue damage (necrosis) were associated with markedly reduced elasticity and viscosity visualized *in vivo*. This relation is irrespective of the collagen fraction within the necrotic regions and explains the heterogeneous appearance of the viscoelastic maps, as well as the absence of spatial correspondence with the homogenous network of collagen in the luc-PANC-1 tumors, which exhibit marked and widespread regions of multifocal discrete necrosis. This is also consistent with previous MR elastography studies reporting a reduction in viscoelastic properties following vascular targeted-therapy induced cell death and/or reduction in

1 vascular density (18,20,21). Whilst this demonstrates the importance of both a viable cellular  
2 and vascular network to tissue integrity and mechanopathology, our data show they provide  
3 a very moderate contribution to the observed range of mechanical phenotypes, in contrast to  
4 differences in ECM characteristics.

5  
6 Intracranial tumors were typically at the softer end of the spectrum of viscoelastic properties  
7 measured in this study ( $G_d \sim 4\text{kPa}$  and  $G_l \sim 2\text{kPa}$ ) (19,22). The high compliance (inversely  
8 related to elastic modulus) of human brain tumors relative to the surrounding brain  
9 parenchyma has been reported, and in the case of glioblastoma, stiffness has been shown  
10 to decrease with tumor grade, measured as part of clinical MR elastography-embedded  
11 prospective studies (44,45). Brain tumors share the unique composition of the healthy brain,  
12 characterized by the absence of a fibrillar network (which resists shear deformation) and a  
13 reliance on hygroscopic hyaluronic acid (the main mechanical support against compressive  
14 forces that act to cause a change in volume but offer little resistance to shear) for  
15 mechanical support. The lack of collagen, a major facilitator of tumor cell intravasation, one  
16 of the earliest stages of metastasis, is consistent with the fact that intracranial tumors rarely  
17 disseminate outside the brain (46). Note also the relatively low viscoelastic properties of the  
18 intracranially grown luc-MDA-MB-231 LM2-4 tumors compared to those propagated in the  
19 mammary fat pad, highlighting the contribution of implantation site to the resulting  
20 biomechanical phenotype. Abdominal neuroblastomas spontaneously arising in Th-MYCN  
21 transgenic mice, and which are derived from the sympathetic nervous system, were also  
22 relatively soft with little collagen, consistent with their general clinical presentation (47).  
23 Interestingly, increased collagen III (reticulin) deposition helps to define an ultra-high-risk  
24 group of patients in which increased stiffness relates to metastatic potential, the major cause  
25 of mortality for children with neuroblastoma (48,49).

26  
27 MR elastography revealed an acute reduction in breast tumor elasticity and viscosity  
28 following systemic administration of collagenase. A similar biomechanical response was  
29 recently reported following intratumoral injection of collagenase, measured using US-based  
30 elastography (16). Collagenase has also been shown to rapidly decrease tumor IFP in  
31 breast cancer xenografts (50,51). Cleavage of collagen with collagenase produces large  
32 peptide fragments which remain trapped within the ECM, making the acute effects of  
33 collagenase challenging to detect on conventional histology at such an acute time point (12).  
34 Having established their dependence on relative tumor collagen fraction, quantitation of  
35 tumor  $G_d$  and  $G_l$  can also provide early MRI biomarkers of response to collagen degradation  
36 *in vivo*. In this way, MR elastography could thus be exploited for monitoring direct enzymatic  
37 degradation of the ECM and/or targeted inhibition of collagen synthesis, potent strategies



1 being actively investigated to improve drug penetration in solid tumors, and showing  
2 promising results in clinical trials (10,12,52).

3

4 Solid stress has been shown to correlate with collagen deposition and be the major  
5 contributor to total tumor pressure in models of pancreatic cancer (53). ECM stiffening is a  
6 marker of poor prognosis in breast cancer and pancreatic ductal adenocarcinoma  
7 (6,37,38,40). Given its sensitivity to collagen deposition, MR elastography may thus  
8 potentially provide prognostic information, and through its sensitivity to collagen modulation,  
9 provide biomarkers of response to collagen-targeted approaches designed to alleviate solid  
10 stress for improved drug delivery. Tumor shear modulus, measured by US elastography, has  
11 been shown to positively correlate with collagen deposition and inversely correlate with  
12 functional vasculature and drug delivery (17). However, MR elastography cannot measure  
13 pressure directly, and as such may not be directly informative for strategies designed to  
14 decrease fluid stress (e.g. hyaluronidase), for which direct measurements of IFP are  
15 required. Reconstruction of tumor viscoelastic parameters from the properties of propagating  
16 waves or strain visualized by MRI or US has often used a simple monophasic linear  
17 viscoelastic mechanical model, which does not take in account any mobile fluid component  
18 (54). Measuring IFP and solid stress in itself represents a major clinical challenge, as current  
19 approaches for measuring both are invasive. Clinical measurement of IFP with wick-in-  
20 needles only permits very discrete sampling of the tissue and is unreliable, whilst innovative  
21 pre-clinical MRI methods shown to correlate with IFP *in vivo* would be difficult to routinely  
22 implement in a clinical setting (51,55).

23

24 Finally, the utility of MR elastography is being actively evaluated clinically in a wide range of  
25 health conditions including neuro- and cardiovascular pathologies, with applications  
26 including diagnosis, staging, surgical planning and intraoperative guidance. For example, the  
27 integration of MR elastography into the management of patients with chronic liver disease is  
28 now becoming well established for its excellent diagnostic accuracy, superior to that of US-  
29 based techniques, and ability to discriminate the different stages of liver fibrosis,  
30 characterized by increased deposition and crosslinking of collagen (56). Whilst less widely  
31 available and more costly than US-based techniques, MR elastography does allow the three  
32 dimensional investigation of large tissue areas and deep-seated organs. MR elastography  
33 can uniquely afford the non-invasive investigation of the mechanical properties of the brain  
34 and its pathology, including as recently demonstrated, neuronal activity (57). Lastly, MR  
35 elastography can be incorporated into multiparametric MR imaging protocols to enable  
36 comparison with other MRI-derived biomarkers of tumor structure and function in a single  
37 clinical scanning session.

1

2 In conclusion, we have shown that quantitation of elasticity  $G_d$  and viscosity  $G_l$  are sensitive  
3 imaging biomarkers of tumor collagen deposition, and response to direct enzymatic  
4 degradation of the collagen network. Given the importance of elevated ECM stiffness in  
5 tumor progression, and the continuing need for new technologies for faster and more  
6 accurate detection, diagnosis and monitoring, MR elastography has the potential to inform  
7 non-invasively on prognosis and improve risk stratification of cancer patients with dense  
8 stroma, and accelerate the development of stromal targeting/modulating treatment  
9 strategies.

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1 **Table 1. Summary of the *in vivo* models**

							2
Cell line	Characteristics	Study	Injection route	cells injected	n	Tumor volume (mm <sup>3</sup> )*	
<b>luc-MDA-MB-231 LM2-4</b>	Highly malignant human triple negative breast cancer cells isolated from a lung metastasis	MRE-histology correlation	<b>i.c.</b>	5 x 10 <sup>3</sup>	5	29 ± 8	
		MRE-histology correlation	<b>o.t.</b>	2 x 10 <sup>6</sup>	6	406 ± 47	
<b>luc-RG2</b>	Rat glioma cells	MRE-histology correlation	<b>o.t.</b>	5 x 10 <sup>3</sup>	5	30 ± 4	
<b>luc-U-87 MG</b>	Human glioblastoma cells	MRE-histology correlation	<b>i.c.</b>	5 x 10 <sup>4</sup>	8	26 ± 4	
		MRE-histology correlation	<b>s.c.</b>	3 x 10 <sup>6</sup>	6	616 ± 114	
<b>luc-D-212 MG</b>	Derived from a pediatric hemispheric giant-cell glioblastoma	MRE-histology correlation	<b>i.c.</b>	1.5 x 10 <sup>5</sup>	7	12 ± 3	
<b>MDA-MB-231</b>	Highly malignant human triple negative breast cancer cells	Collagenase treatment	<b>o.t.</b>	2 x 10 <sup>6</sup>	14	385 ± 49	
<b>BT-474</b>	Invasive ductal breast carcinoma cells	MRE-histology correlation	<b>o.t.</b>	5 x 10 <sup>6</sup>	7	438 ± 36	
		Collagenase treatment	<b>o.t.</b>	5 x 10 <sup>6</sup>	7	287 ± 50.8	
<b>luc-PANC-1</b>	Pancreatic epithelioid carcinoma cells	MRE-histology correlation	<b>o.t.</b>	1 x 10 <sup>7</sup>	7	1402 ± 280	
<b>Transgenic mouse models</b>							
<b>Th-MYCN</b>	High-risk neuroblastoma	MRE-histology correlation	Spontaneously arising tumors		5	1369 ± 254	
<b>GTML/Trp53<sup>KO</sup></b>	Medulloblastoma	MRE-histology correlation	Spontaneously arising tumors		7	27 ± 4	

3 \*Tumor volumes at the time of the MR elastography experiment and determined using segmentation from regions  
 4 of interest drawn on each tumor-containing T<sub>2</sub>-weighted MRI slice. i.c. intracranial, o.t. orthotopic, s.c.  
 5 subcutaneous  
 6

7



1 **Figure legends**

2

3 **Figure 1. MR elastography reveals marked differences in viscoelasticity measured *in***  
4 ***vivo* in orthotopic and transgenic models of cancer.** (A) Representative  $T_2$ -weighted  
5 MRI and associated parametric maps of elasticity  $G_d$  and viscosity  $G_l$  obtained from a  
6 medulloblastoma spontaneously arising in the brain of a GTML/*Trp53*<sup>KI/KI</sup> transgenic mouse  
7 (n=7), intracranially (i.c.) propagated luc-MDA-MB-231 LM2-4 breast cancer (n=5) and luc-D-  
8 212-MG glioblastoma xenografts (n=7), a neuroblastoma spontaneously arising in a Th-  
9 *MYCN* transgenic mouse (n=5), intracranially propagated luc-RG2 (n=5) and luc-U-87 MG  
10 glioblastoma xenografts (n=8), and orthotopic BT-474 breast cancer (n=7), luc-PANC1  
11 pancreatic cancer (n=7) and luc-MDA-MB-231 LM2-4 breast cancer (n=6) xenografts. The  
12 dashed line (---) indicates the tumor boundaries defined on  $T_2$ -weighted images. (B)  
13 Quantitative summary of mean of the mean tumor  $G_d$  and  $G_l$  ( $\pm 1$  s.e.m) for each model.  
14 The insets show that the collective average of  $G_d$  and  $G_l$  determined in the breast and  
15 pancreatic tumors was significantly higher than in tumors arising in the nervous system  
16 (\*\*\*\* $p < 0.0001$ ).

17

18 **Figure 2. MR elastography can inform on tumor stromal modulation induced by**  
19 **collagenase.** (A) Representative  $T_2$ -weighted anatomical MRI images, and parametric  
20 maps of  $G_d$  and  $G_l$ , acquired from mice bearing orthotopic BT-474 breast cancer xenografts  
21 24 hours prior to and 5 hours after intravenous injection of either vehicle or collagenase. (B)  
22 Relative changes (%) in tumor median  $G_d$  and  $G_l$  measured in orthotopic MDA-MB-231 (blue  
23 symbols) and BT-474 (red symbols) breast cancer xenografts measured 5 hours after  
24 administration of either vehicle (O) or collagenase ( $\Delta$ ). Data are the individual changes  
25 from each tumor, and the combined cohort mean  $\pm 1$  s.e.m. Collagenase induced a  
26 significant reduction in both  $G_d$  and  $G_l$  compared to vehicle control.

27

28 **Figure 3. MR elastography-derived elasticity  $G_d$  and viscosity  $G_l$  correlate with tumor**  
29 **collagen fraction.** Representative anatomical  $T_2$ -weighted MRI images and parametric  
30 maps of elasticity ( $G_d$ ) and viscosity ( $G_l$ ), and the corresponding computed maps of  
31 picrosirius red staining (collagen I & III), hematoxylin and eosin staining (cellularity) and  
32 immunohistochemical detection of Cd31 (vascular density) extracted from high-resolution  
33 images of tissue sections from: (A) a medulloblastoma spontaneously arising in the brain of  
34 a GTML/*Trp53*<sup>KI/KI</sup> transgenic mouse, a neuroblastoma spontaneously arising in a Th-*MYCN*

1 transgenic mouse, and an intracranially propagated luc-U87-MG glioblastoma xenograft, and  
2 **(B)** from orthotopic BT-474, luc-PANC-1 and luc-MDA-MB-231 LM2-4 breast and pancreatic  
3 cancer xenografts. Note that the areas above the brain tumors showing both high  
4 viscoelastic properties and high picosirius red staining correspond to regions where the  
5 tumor has invaded the collagen-rich meninges. Dotted lines indicate regions of necrosis.  
6 Note that in necrotic areas, high collagen density is not associated with high value of  $G_d$  and  
7  $G_l$ . **(C)** Scatter graphs of individual tumor mean  $G_d$  and  $G_l$  plotted against mean collagen  
8 fraction (%), cellularity and vessel density. Linear regression analysis and 95% confidence  
9 intervals for significant correlations are shown.

10

11 **Figure 4. The relationship between MR elastography-derived tumor viscoelastic**  
12 **properties and the spatial distribution and deposition of collagen evaluated by texture**  
13 **analysis.** Scatter graphs of individual mean tumor elasticity  $G_d$  and viscosity  $G_l$  plotted  
14 against **(A)** entropy and **(B)** fractal dimension (FD). Linear regression analysis and 95%  
15 confidence intervals for significant correlations are shown. Note that both  $G_d$  and  $G_l$  are  
16 negatively correlated with entropy and positively correlated with FD, indicating that increased  
17 tumor stiffness is associated with more complex and more homogeneously distributed  
18 collagen. Entropy values close to zero and relatively high values of FD determined in the BT-  
19 474, luc-PANC-1 and luc-MDA-MB-231 LM2-4 tumors are consistent with the presence of  
20 such a homogeneous and dense collagen network. **(C)** Scatter graphs showing the mono-  
21 exponential relationship between entropy and collagen fraction ( $y= 3.86e^{-0.24x}$ ,  $r^2=0.76$ ), and  
22 logarithmic relationship between fractal dimension and collagen fraction ( $y= 0.15\ln x + 1.16$ ,  
23  $r^2=0.98$ ).

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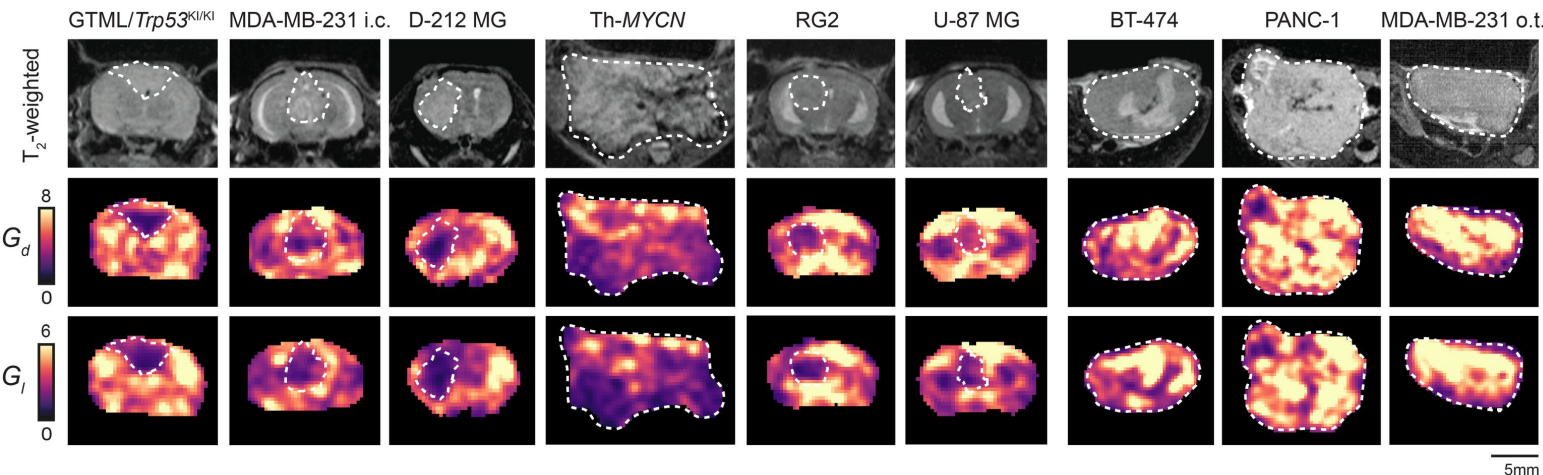


Figure 1

A

Tumors arising in the nervous system

Breast and pancreatic tumors



B

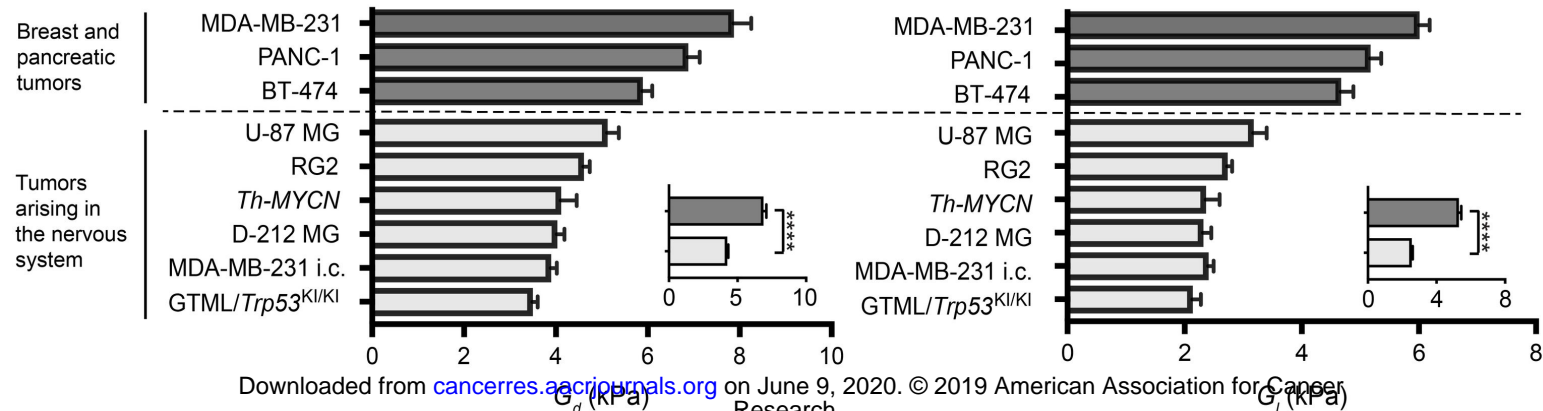
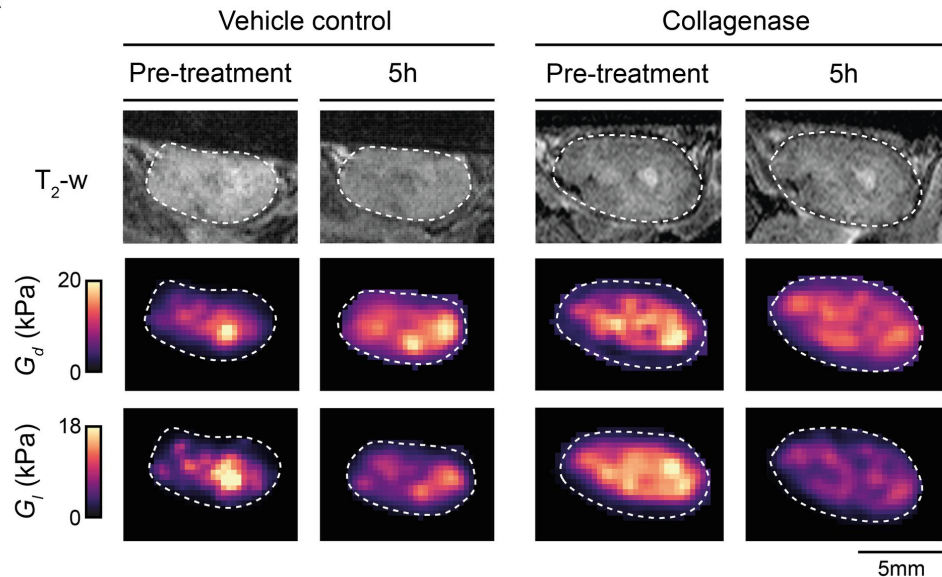


Figure 2

**A**



**B**

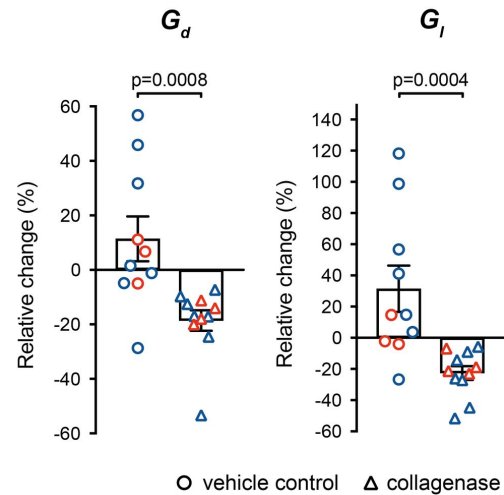


Figure 3

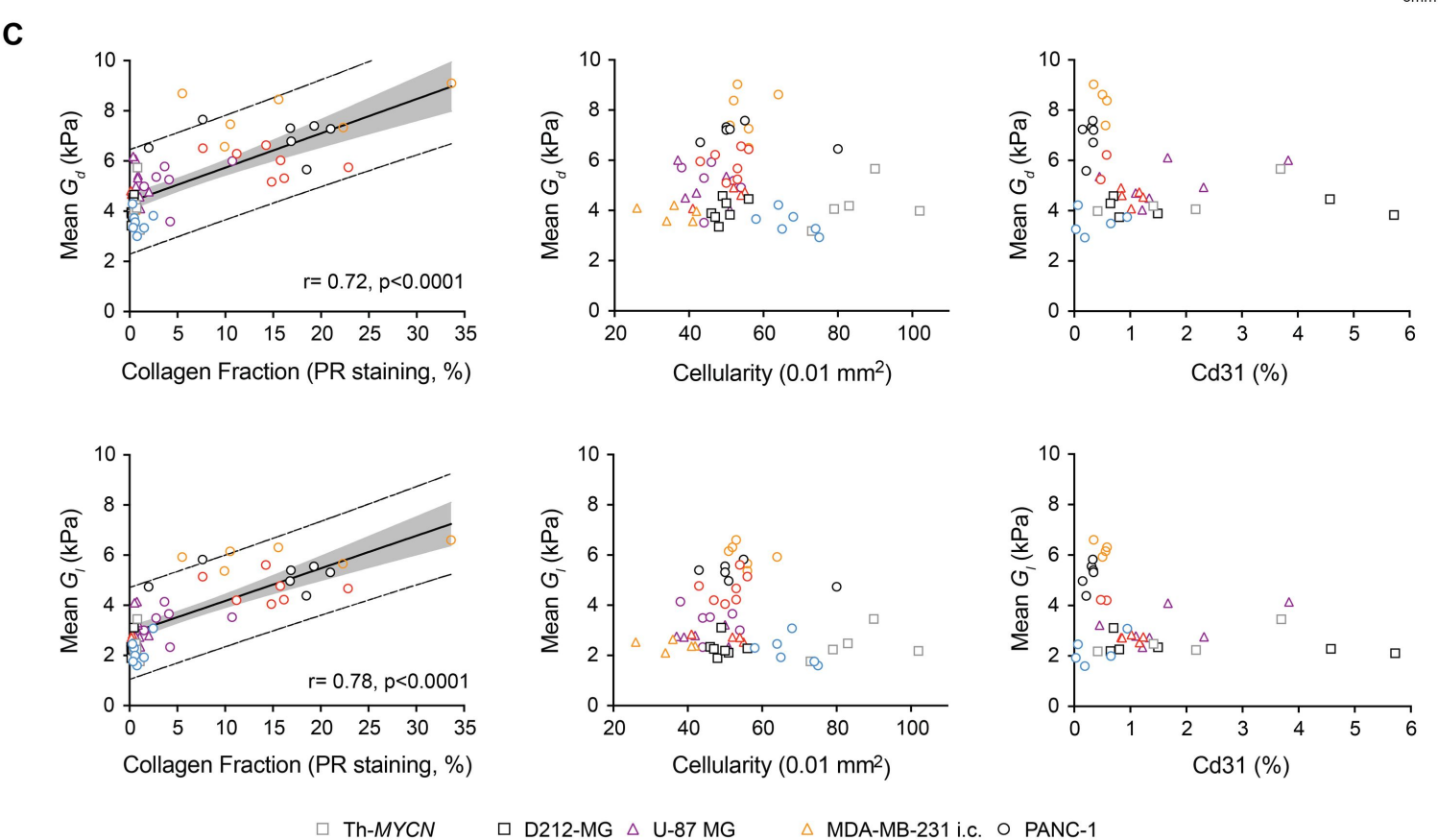
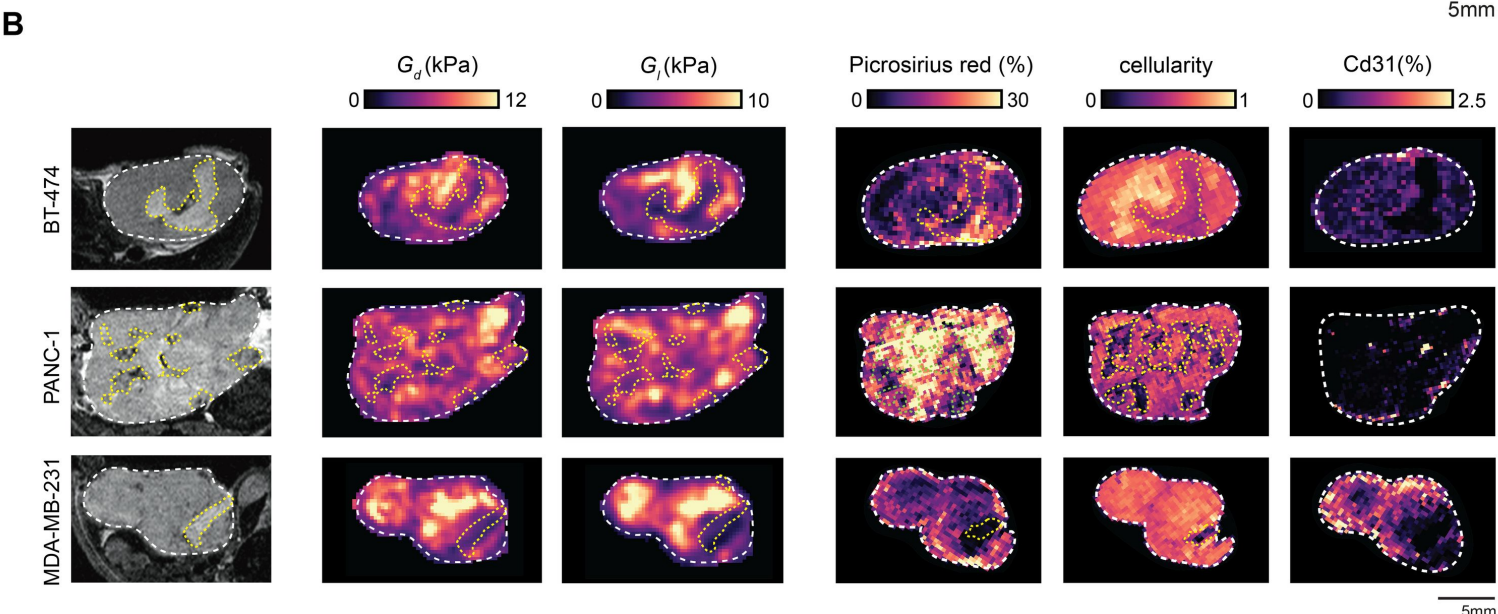
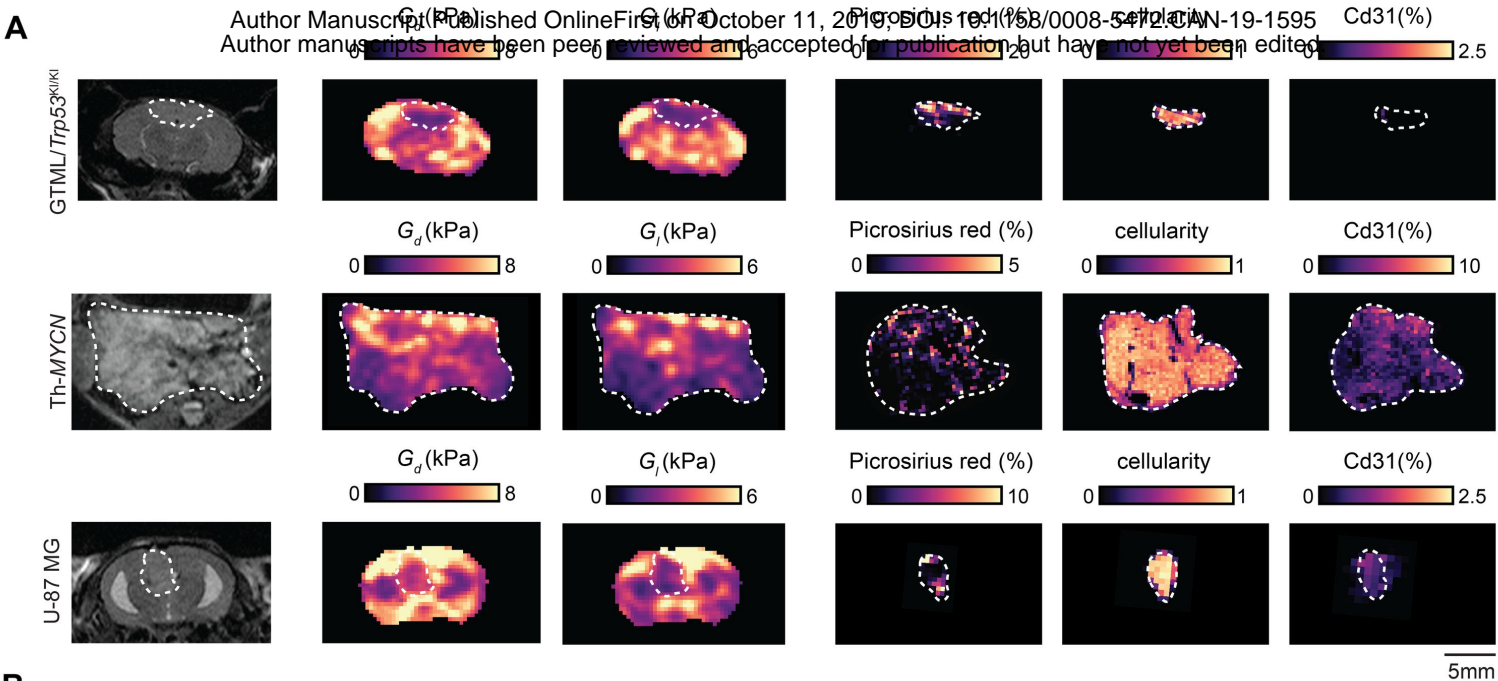
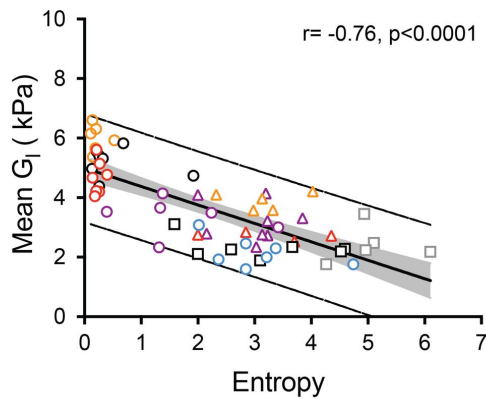
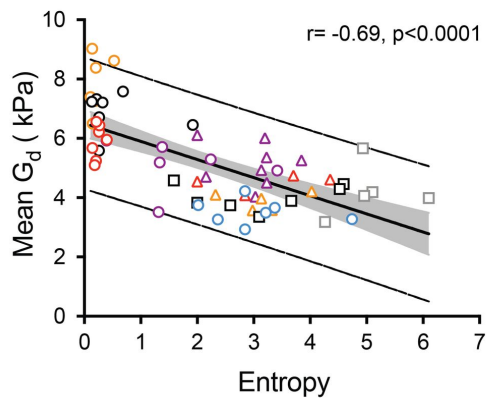
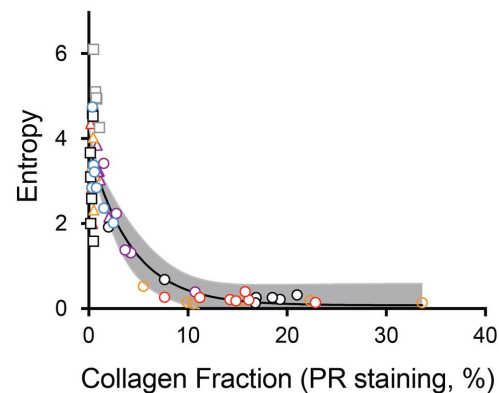


Figure 4

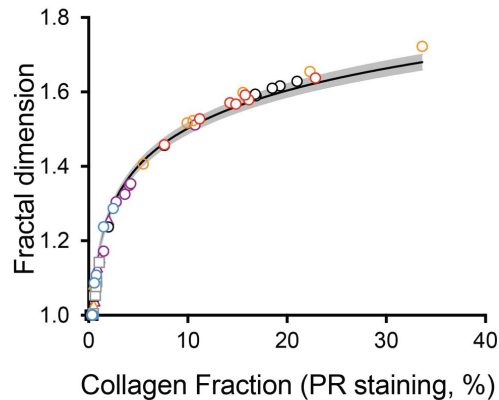
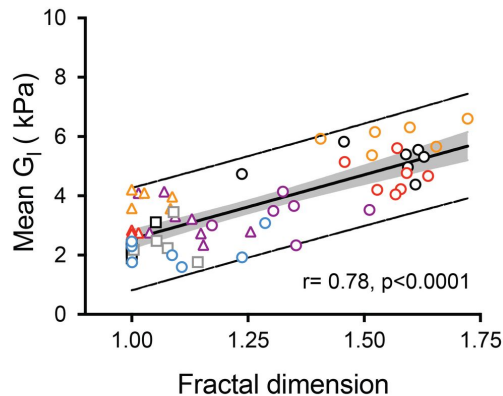
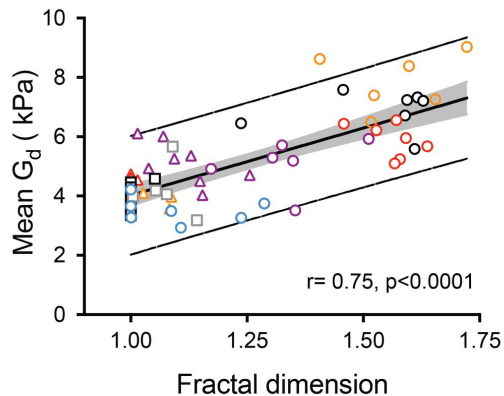
**A**



**C**



**B**



□ Th-MYCN    □ D-212 MG    △ U-87 MG    △ MDA-MB-231 i.c.    ○ PANC-1

# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## Investigating the Contribution of Collagen to the Tumor Biomechanical Phenotype with Non-invasive Magnetic Resonance Elastography

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