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## Original Article

# Assessing tumor vascularization as potential biomarker of imatinib resistance in Gastrointestinal Stromal Tumors by Dynamic Contrast-Enhanced Magnetic Resonance Imaging

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**Short title: DCE-MRI estimates GIST tumor vascularization**

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## **Abstract**

**Background:** Most metastatic gastrointestinal stromal tumors (GISTs) develop resistance to the first line imatinib treatment. Recently, increased vessel density and angiogenic markers were reported in GISTs with worse prognosis, suggesting the angiogenesis implication in GIST tumor progression and resistance. The purpose of this study is to investigate the relationship between tumor vasculature and imatinib resistance in different GIST mouse models by a non-invasive magnetic resonance imaging (MRI) functional approach.

**Methods:** Immunodeficient mice (n=8 for each cell line) were grafted with imatinib-sensitive (GIST882 and GIST-T1) and resistant (GIST430) human cell lines. Dynamic contrast-enhanced MRI (DCE-MRI) were performed on GIST xenografts to quantify tumor vessel permeability ( $K^{\text{trans}}$ ) and vascular volume fraction ( $v_p$ ). Microvessel density (MVD), permeability (mean dextran density, MDD) and angiogenic markers were evaluated by immunofluorescence and western blot assays.

**Results:** DCE-MRI showed significantly increased vessel density ( $p < 0.0001$ ) and permeability ( $p = 0.0002$ ) in imatinib-resistant tumors compared to imatinib-sensitive ones. Strong positive correlation is observed between MRI estimates,  $K^{\text{trans}}$  and  $v_p$ , and their related *ex-vivo* values, MVD ( $r = 0.78$  for  $K^{\text{trans}}$  and  $r = 0.82$  for  $v_p$ ) and MDD ( $r = 0.77$  for  $K^{\text{trans}}$  and  $r = 0.94$  for  $v_p$ ). In addition, higher expression of vascular endothelial growth factor receptors (VEGFR2 and VEGFR3) is shown in GIST430.

**Conclusions:** DCE-MRI highlights marked differences in tumor vasculature and microenvironment properties between imatinib-resistant and sensitive GISTs, confirmed by *ex-vivo* assays. These results provide new insights in the role that DCE-MRI may play for GIST characterization and response to treatment. Validation studies are warranted to confirm these findings and determine potential implications.

**Miniabstract**

DCE-MRI detects significant increased vascularity and vessel permeability in imatinib-resistant GIST tumors in comparison to sensitive ones. Therefore, vascularization/permeability properties could be investigated as GIST biomarkers of therapy response.

**Keywords**

Gastrointestinal Stromal Tumor; angiogenesis; DCE-MRI; imatinib; Gadolinium contrast agent.

## **Introduction**

Gastrointestinal stromal tumor (GIST) is the most common malignant mesenchymal neoplasm of the digestive tract, with a mean annual incidence of 11-14 patients per million people. Surgical resection is the first line treatment for localized or resectable GISTs. However, every GIST is considered to be potentially malignant and metastases are observed in liver or peritoneal cavity in 50% of cases following primary surgical resection [1, 2]. GISTs are commonly distinguished from other sarcomas by gain-of-function mutations of the tyrosine kinase KIT receptor [3]. Imatinib (Gleevec; Novartis Pharmaceuticals) is a potent inhibitor of KIT and is currently the only effective treatment against metastatic and unresectable GIST [4-7]. However, clinical data highlight imatinib failure in the complete eradication of the disease since most of patients develop resistance after few months of treatment, with significant complications in the follow-up studies [8, 9].

Particular issues rely on the evaluation of GIST prognosis along transformation from benign to malignant tumor. The Fletcher classification system easily allows accurate stratification of GIST patients assessing tumor size, mitotic count and anatomic location [10]. Moreover, recent studies demonstrated the prognostic significance of some molecular markers, as the proliferating cell nuclear antigen Ki-67 and the KIT mutational status [11, 12]. However, the evaluation of most of these GIST signatures can be performed only after surgical resection of the whole tumor or can be biased by the limited sampling of biopsies, with evident obstacles of prognosis for inoperable GISTs.

Notably, several investigations have introduced *ex-vivo* the association of vascularization and angiogenic markers with GISTs presenting the worst prognosis [13-16]. Considering the prognostic role of angiogenesis in GIST, the identification of reliable non-invasive tools able to monitor tumor vascularization may provide new insight in GIST characterization and therapy response evaluation. Solid tumors typically display altered and unstructured

vasculature responsible of irregular perfusion and permeability [17] and these properties can be assessed by several imaging approaches that allow the visualization of intratumoral vessels [18, 19]. Among them, the dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) approach offers the unique advantage of combining high spatial resolution and tissue contrast with functional information [20]. Following the injection of a paramagnetic contrast agent (CA), it is possible to evaluate the tissue contrast enhancement produced by its extravasation through hyperpermeable tumor vessels and extrapolate pharmacokinetic parameters informative of vascular permeability and perfusion ( $K^{trans}$ ), extracellular volume fraction ( $v_e$ ), and blood plasma volume fraction ( $v_p$ ) [21, 22]. DCE-MRI has proven to be a promising tool for the assessment of malignancy in different cancers and the obtained kinetic constants can be exploited as biomarkers to assess tumor angiogenesis and response to antiangiogenic therapy [23-27].

We therefore hypothesized that quantitative permeability measurements might reveal characteristic vascularization properties between imatinib sensitive and resistant GIST tumors. This assumption is supported by studies assessing perfusion in GIST tumors by exploiting contrast enhanced ultrasonography and computed tomography [28, 29]. For this purpose, three mouse models of imatinib-sensitive and resistant tumors on highly immunodeficient NOD scid gamma (NSG) mice were used. Functional MRI was exploited to characterize tumor microenvironment in terms of vascularization and permeability by combining DCE-MRI with a new Gd-based blood pool contrast agent [30].

## **Material and methods**

GIST cell lines culture and MRI image analysis are described in the Supplementary Methods.

### ***Mice***

Male 7-week-old NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ(NSG) mice with an average body weight of 30g were used. All animals were housed in sterile cages under laminar flow hoods in a temperature controlled room with a 12-hour light/12-hour dark schedule and fed with autoclaved chow and water *ad libitum*. Mice were maintained at the animal facility of the Molecular Biotechnology and Health Sciences department at University of Turin and treated in accordance with University Ethical Committee and European guidelines (Directive 2010/63) under the protocol number 0081521.

Heterotopic GIST xenografts were generated by subcutaneous bilateral injection of GIST882, GIST430 and GIST-T1 cell lines. For each cell line, n=8 mice have been bilaterally inoculated. GIST cells were suspended in 50  $\mu$ l of Phosphate Buffered saline (PBS) mixed with 50  $\mu$ l of Matrigel<sup>TM</sup> Matrix (BD Pharmigen, Milano, Italy) at a density of  $2 \times 10^6$ ,  $1 \times 10^6$  and  $2,5 \times 10^4$  for GIST882, GIST430 and GIST-T1, respectively. Tumor growths were weekly monitored over time by using a caliper and tumor volumes calculated by  $[(\text{length} \times \text{width}^2)/2]$ .

### ***In vivo imaging experiments***

T<sub>2</sub>-weighted (T<sub>2w</sub>) MRI anatomical acquisitions have been weekly performed and tumor volumes calculated on images by drawing a region of interest (ROI) for both tumors using ITK-SNAP software (<http://www.itksnap.org/pmwiki/pmwiki.php>).

When tumors reached a volume in the range of 30-500 mm<sup>3</sup>, DCE-MRI experiments were performed. Mice were anesthetized by injecting a mixture of tiletamine/zolazepam (Zoletil 100; Virbac, Milan, Italy) 20 mg/kg and xylazine (Rompun; Bayer, Milan, Italy) 5 mg/kg and a 27-gauge catheter was introduced into the tail veins for contrast agent (CA) injection. MR images were acquired with a 1 Tesla Aspect M2 MRI System (Aspect Magnet Technologies Ltd., Netanya, Israel). T<sub>2w</sub> anatomical images were acquired for monitoring tumor progression with a Fast Spin Echo sequence (TR = 2500 s; TE = 44ms; number of slices = 10; slice thickness = 1.5 mm; FOV = 40 mm; matrix = 152  $\times$  160; NEX = 4; acquisition time = 3 m 20 s).

DCE-MRI dynamic protocol consisted of an axial  $T_{1w}$  3D spoiled Gradient Echo sequence with 3 initial pre-contrast images acquisition followed by the injection of a gadolinium binding serum albumin CA (Gd-AAZTA-MADEC, CAGE Chemicals, Novara, Italy) through the catheter. After injection at a dose of 0.03 mmol Gd/kg b.w., 37 dynamic post-contrast images were acquired with the following parameters: TR = 40; TE = 2.1msec, flip angle = 60°, number of slices = 10, slice thickness = 1.5 mm, FOV = 40 mm, matrix = 128 × 128.

### ***Immunofluorescence staining***

After MRI acquisition, 0.25 mg Dextran-Texas red 40 kD (Life Technologies, Monza, Italy) was intravenously injected in mice to assess vessels leakage. Ten minutes later, mice were sacrificed, tumors excised and embedded in Optimal Cutting Temperature matrix compound (Tissue-Tek® OCT™) for cryosection staining and preserved at -80°C. Texas Red-conjugated Dextran signal was amplified with polyclonal rabbit anti-Texas Red® (Life Technologies, Monza, Italy). MVD was assessed by CD31 staining (monoclonal rat anti-CD31, BD Pharmigen, Milano, Italy). All secondary antibodies are purchased from Life Technologies (Alexa-Fluor®, Monza, Italy). Briefly, slices were incubated with 10% goat serum for 1 hour at room temperature (RT) and then with primary antibodies (dilution 1:200) overnight at 4 °C. After washing in PBS-Tween 0.1%, slices were incubated with secondary antibody (dilution 1:500) for 1 hour RT. Nuclei were stained with DAPI (Sigma Aldrich, Milano, Italy) and slices rinsed with bidistilled water.

### ***Evaluation of MVD and MDD***

Immunohistochemical assessment was performed using an ApoTome fluorescent microscope (Zeiss, Germany). The degree of angiogenesis was determined by calculating the microvessel density (MVD) on CD31 positive slices and the extravasation of dextran as mean dextran density (MDD). Microvessels were visualized as lumen-containing structures in which all single cells or cluster of cells are positive for CD31 immunereaction. Staining for CD31 was visualized as a green signal (wavelength 488 nm, FITC green), whereas dextran



extravasation emits in red (wavelength 568 nm, Texas red). The entire section was systematically scanned at x100 magnification, ten fields were viewed at x200 magnification and images of CD31, dextran and DAPI (wavelength 461 nm, blue) staining were taken. Two or more positive foci belonging to the same continuous vessel were counted as one microvessel, as described by Weidner et al. [31]. The MVD and MDD were manually counted and averaged over ten fields.

### ***Western blot samples and analysis***

Cells from GIST 430-, 882- and T1-derived tumors were extracted with RIPA buffer (1% Triton X-100, 0,1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 7, 0.4 mM Na<sub>3</sub>VO<sub>4</sub>, inhibitor mix). Cell lysates were centrifuged at 13,000 g for 10 minutes and the supernatants were collected and assayed for protein concentration with the Bio-Rad protein assay method (Biorad, Hercules, CA, USA). Total cell lysates were separated by SDS-PAGE under reducing conditions, transferred to nitrocellulose and immunoblotted overnight with primary antibodies against Vinculin (loading control), VEGFR2 and VEGFR3 at 4 °C. Mouse monoclonal antibody against Vinculin was produced at Molecular Biotechnology Center (MBC), while antibodies against VEGFR2 and VEGFR3 were purchased from Cell Signaling (Beverly, MA,USA). Blots were incubated with mouse or rabbit horseradish-peroxidase conjugated secondary antibodies for 1 h at room temperature. ECL (Euroclone) was used to detect chemoluminescent signals. Protein band intensities were measured by a scanning densitometer (Quantity One; PDI Inc, New York, USA).

### ***Statistical analysis***

Statistical analysis of imaging data, microvessels counting and western blot densitometry was performed using GraphPad Prism 5 software (GraphPadInc, San Diego, California, USA). All data are shown as mean ± SEM. One-way ANOVA analysis and Dunn's multiple comparison tests were used to compare the functional mean MRI-based estimates values obtained in GIST882, -T1 and 430.

One-way ANOVA analysis and Bonferroni multiple comparison tests were performed to evaluate statistical MVD and MDD differences among GIST tumors. The relationship between the *ex-vivo* histological markers of vascularization MVD/MDD and the estimates obtained by DCE-MRI analysis ( $K^{trans}$  and  $v_p$ ) have been assessed with parametric Pearson's rank correlation ( $r$ ). For all tests, a P-value  $<.05$  was considered statistically significant.

## Results

### ***Generation of imatinib-sensitive and resistant GIST models on NSG mice***

GIST882, GIST -T1 and GIST430 cell lines have been subcutaneously inoculated in NSG mice to generate imatinib-sensitive and resistant GIST models. Solid tumors efficiently developed in all the animals considered for the study, exhibiting different kinetic growth related to the inoculated GIST cell lines (Fig. 1a). Palpable masses are typically detected from 15 to 18 days after inoculation. GIST-T1 tumors display fast growth rate, similarly to GIST430. Conversely, tumor growth is much slower in GIST882, reaching a maximum of 400 mm<sup>3</sup> after 45 days from inoculation. Mouse models exhibit different morphological features. Coronal  $T_{2w}$  MRI images highlight highly hemorrhagic and bleeding lesions in GIST-T1 tumors (Fig. 1b), partially similar to what observed for GIST430 tumors. Conversely, GIST882 tumors exhibit dense and compact tissue, with no signs of bleedings. Biopsies and histological H&O results showed substantial morphological differences among GIST models, confirming MRI findings. Cellular and subcellular structures identified by H&O staining are in accordance with those previously reported elsewhere, for all three GIST tumors [32, 33]. Different KIT expression levels among GIST-T1, 882 and 430 tumors were found by Western Blot analysis (Fig. 1c).

### ***DCE-MRI identifies differences in vascularization and permeability between imatinib-sensitive and resistant GIST tumors***

Functional MRI acquisitions were performed on GIST tumors with volumes in the range of 30-500 mm<sup>3</sup>. Tumor microvessels permeability ( $K^{trans}$ ) and plasmatic volume ( $v_p$ ) values have

been calculated by applying a two-compartment pharmacokinetic model on DCE-MR images following the administration of Gd-AAZTA-MADEC, a new blood-pool contrast agent (Figure 2a). Imatinib-resistant GIST430 tumors exhibit significantly higher  $K^{\text{trans}}$  mean values compared to imatinib-sensitive GIST882 and -T1 ( $38.1 \pm 7.4 \text{ E-5}$  for GIST-430,  $14.9 \pm 2.2 \text{ E-5}$  for GIST882 and  $9.8 \pm 2.0 \text{ E-5}$  for GIST-T1,  $P=.0002$ , 1-way ANOVA). A similar trend was observed for  $v_p$ , whose values are significantly higher in the imatinib-resistant tumors compared to sensitive ones ( $0.10 \pm 0.01$  for GIST430,  $0.04 \pm 0.004$  for GIST882 and  $0.02 \pm 0.004$  for GIST-T1;  $P<.0001$ , 1-way ANOVA). Imatinib-sensitive tumors (GIST882 and -T1) show similar mean values for both estimates, without any significant difference. Representative parametric maps of  $K^{\text{trans}}$  and  $v_p$  are overlaid on  $T_2$ -weighted anatomical images and displayed in Fig 2b. Qualitatively, these maps illustrate a substantial increase in  $K^{\text{trans}}$  and  $v_p$  values in GIST430 tumors in comparison to GIST882 and GIST-T1.

### ***Ex vivo evaluation of tumor angiogenesis correlates with MRI quantitative parameters***

Ex-vivo staining for CD31 and dextran was performed to evaluate GIST vascularization and permeability, respectively. Quantitative analysis demonstrated that GIST430 tumors are highly vascularized, with mean MVD= $31.9 \pm 4.6$  (Fig. 3a). Conversely, imatinib-sensitive GIST tumors display lower MVD (MVD=  $18.0 \pm 0.9$  for GIST 882, MVD= $4.9 \pm 0.6$  for GIST-T1). One-way ANOVA analysis shows significant difference in MVD mean values between GIST430 towards GIST-T1 and GIST882 tumors ( $P=.0002$ ). Representative immunofluorescence images of GIST-T1, 882 and 430 show different vascularization levels in GIST-T1, 882 and 430 tumor sections (Fig. 3b). Interestingly, MVD show a strong positive correlation with both DCE-MRI estimates  $v_p$  ( $P<.0001$ ,  $r=0.82$ ) and  $K^{\text{trans}}$  ( $P=.002$ ,  $r=0.78$ ) (Fig. 3c). The extravasation of dextran was assessed to evaluate functional vessel permeability ex-vivo as mean dextran density (MDD, Figure 4a). MDD is more than three-times higher in GIST430 ( $15.7 \pm 2.6$ ) than in GIST882 and -T1 ( $2.7 \pm 0.3$  for GIST882 and  $3.4 \pm 0.3$  for GIST-T1), with statistical significance ( $P=.0003$ , Fig 4a). Representative images of dextran-Texas red

extravasation and CD31-positive vessels are shown in fig 4b. Strong positive correlation is found between MDD and  $K^{trans}$  ( $P=.006$ ,  $r=0.77$ ) and MDD and  $v_p$  ( $P=.0001$ ,  $r=0.94$ , Fig. 4c). Expression of endothelial receptors involved in tumor angiogenesis (VEGFR2) and lymphoangiogenesis (VEGFR3) was investigated by western blot analysis in GIST tumors as additional markers of tumor angiogenesis (Fig. 5a-b). Both VEGFR2 and VEGFR3 display a greater than three-fold increase in terms of expression level in GIST430 compared to GIST-T1 and 882 ( $P=.0015$  for VEGFR2,  $P=.0007$  for VEGFR3).

## Discussion

The aim of our work was to evaluate the ability of a functional MRI-based approaches to highlight differences in tumor microenvironment properties related to imatinib resistance in GIST murine models. For this purpose, GIST tumor vascularization was investigated by a DCE-MRI approach. Our findings demonstrated that DCE-derived pharmacokinetic parameters can detect differences in plasmatic volume and permeability among the investigated GIST tumor cell lines. In particular, higher  $K^{trans}$  and  $v_p$  values were measured for the imatinib-resistant tumors (GIST430), compared to both imatinib-sensitive ones (GIST-T1 and GIST882). This study indicates that the characterization of tumor microenvironment and vasculature properties of GIST tumors by a functional MRI-based approach can discriminate between imatinib-responsive and -resistant tumors.

Angiogenesis is one of the fundamental steps for the progression and metastasis of solid tumors. Clinical implications of angiogenesis and its prognostic significance have been reported in several cancers, also in lesions of the gastrointestinal tract [34-37]. Despite this, the risk of recurrence or the metastatic potential in GIST is commonly predicted by the Fletcher classification system, mainly based on the evaluation of anatomical criteria. Only recently, several factors involved in the angiogenesis process have been proposed as additional predictive biomarkers of GIST transition from benign to malignant lesions. *Ex-vivo*

studies on GIST specimens showed that MVD is closely related to VEGF expression and strongly associated with GIST with poorer prognosis. These data pointed out that angiogenesis and vascularization are associated with tumor grade, mitotic count and higher risk of metastasis in GIST [15, 16]. Further confirming the key role that angiogenesis plays in GIST pathogenesis, we show that imatinib-sensitive and -resistant tumors have different vascularization properties by exploiting a DCE-MRI approach.

In particular, GIST430 tumors exhibit more than two-fold higher  $K^{\text{trans}}$  and  $v_p$  values if compared to imatinib-sensitive tumors, with statistical significance. DCE-MRI results identified a more unstructured and deregulated vasculature in terms of blood flow and permeability ( $K^{\text{trans}}$ ) and vascular density ( $v_p$ ), properties linked to the angiogenic process. Moreover, our *in vivo* functional findings are validated by histological quantification of endothelial vessels (CD31) and permeability (dextran). Both parameters confirmed higher vascularization and permeability in GIST430 tumor sections, compared to GIST-T1 and 882 ones. These findings are in accordance with recent data showed by Imamura et al [13], where higher expression of VEGF and increased MVD were found in GIST tumor harboring KIT mutation associated with resistance to imatinib. However, so far little is known about the association between angiogenesis and imatinib resistance in GIST. To the best of our knowledge, this is the first study that investigated tumor vascularization properties in several GIST metastatic murine models and detected functional differences in imatinib-resistant and -sensitive tumors by exploiting the *in vivo* DCE-MRI approach. Interestingly, a significant positive correlation was found between  $v_p$  and MVD. The higher  $v_p$  observed in the imatinib-resistant tumors indicates a larger vascular space; this result is confirmed by the higher MVD values in GIST430, suggesting that  $v_p$  can be used as a marker of vessel density. In particular, considering the role of MVD in GIST prognosis, we could hypothesize that  $v_p$  could be used to assess *in vivo* the GIST aggressiveness. GIST882 showed significantly higher MVD in comparison to GIST-T1, whereas any difference is detected by DCE-MRI. We can explain this mismatch considering that  $K^{\text{trans}}$  and  $v_p$  estimates assess only functional vessels, whereas MVD assesses vessels independently of their functionality. Consequently, DCE-

MRI can detect poor perfusion properties of GIST882 tumors despite the presence of a relatively high vessel density.

Moreover, Yamashita et al.[38] observed *in vivo* intratumoral vessels in GIST patients with worst prognosis by contrast-enhanced ultrasound technique. The visualization of intratumoral vessels correlated with VEGF expression, highlighting the relationship between angiogenesis and malignancy in GIST. Interestingly, we observed increased expression of VEGFR2 and VEGFR3 in GIST430 tumors in comparison to GIST882 and GIST-T1. VEGFR2 plays a well-known role in tumor angiogenesis formation and sprouting, whereas VEGFR3 is mainly involved in the lymphoangiogenesis process. Lymphoangiogenesis promotes and sustains tumor progression and angiogenesis and favors the metastases spread-out through the surrounding lymphatic networks. Several studies indicate that VEGFR3 is usually highly expressed in the most aggressive human cancers [39-41]. Our findings suggest that, in addition to VEGFR2, VEGFR3 expression may be relevant for GIST imatinib-resistant tumor growth and the direct targeting of these receptors could be promising in non-responding GIST tumors.

Second and third lines of GIST therapeutic regimen following imatinib resistance consider antiangiogenic targeted therapies as a key factor for alternative treatment options. Sunitinib is a multityrosine kinase inhibitor that is clinically approved for the treatment of imatinib-resistant GIST [42]. In addition to KIT and PDGFR, sunitinib targets also VEGFR1 and VEGFR2 receptors. Recently, Kim et al. [43] reported in a pilot clinical study that the effect of sunitinib treatment in GIST patients can be quantitatively monitored by DCE-MRI. In particular, they observed significant reduction in  $K^{trans}$  values upon therapy that can be explained by the reduced wash-in rate following vessel density reduction. Their study, although limited to few patients, showed that the DCE-MRI technique can detect vascular functional changes in treated GIST and that it can be exploited as a valid alternative to conventional treatment assessment approaches. In addition, perfusion imaging studies based on contrast enhanced computed tomography demonstrated lower perfusion values in

good responders, whereas poor responders showed significant lower perfusion values [44, 45]. Our study clearly demonstrated in GIST mouse models that imatinib-resistant tumors exhibit higher  $K^{trans}$  and  $v_p$  values compared to imatinib-sensitive ones. Therefore, changes in these functional properties can be non-invasively monitored by DCE-MRI to assess the efficacy of antiangiogenic treatments, both for sunitinib, as for other specific drugs that are currently in Phase II or III of clinical trials [46].

Several findings suggest that the evaluation of tumor neovascularity and associated permeability changes can be improved by using macromolecular Gd-based CA adducts, that can accumulate within the tumor owing to the enhanced permeability and retention effect [47]. The main advantages rely in a better assessment of the tumor vascularization properties in comparison to small molecular weight CAs, in combination with higher contrast efficiency at low-intermediate magnetic fields (0.5-1.5 T) [48-50]. Our results, obtained using a new Gd-based blood pool CA [30], demonstrate that accurate characterization of GIST tumor microvascular properties is feasible at low magnetic fields, hence facilitating translational purposes.

This study presents some limitations. First, only GIST cell lines sensitive or resistant to imatinib have been investigated. Further studies are needed to extend the herein reported evidences to GIST cell lines sensitive or resistant to other TK inhibitors (e.g. sunitinib or regorafenib). Moreover, a small number of GIST cell lines have been investigated. Mice were inoculated with two imatinib-sensitive (GIST-T1 and GIST882) and one imatinib-resistant (GIST430) cell lines. Further evaluations on additional GIST cell lines and on patient-derived tumors could extend the proposed MRI approaches for characterizing GIST tumors and for assessing novel TK inhibitors [51, 52]. However, GIST-T1, GIST882 and GIST430 cells are the most used and well characterized GIST cell lines, and commonly considered representative of imatinib-sensitive and resistant tumors. An additional limitation relies on the fact that clinical trials are needed to confirm DCE-MRI as a valuable tool in assessing GIST tumor treatment response.

In conclusion, our work highlights the important role that functional MRI approaches can provide to detect functional differences between imatinib-sensitive and -resistant GIST tumors. In particular, changes in microvessel permeability and density among GIST tumors are pointed out by our DCE-MRI approach. Imatinib-resistant tumors exhibit increased  $K^{trans}$  and  $v_p$  values compared to imatinib-sensitive ones, as confirmed by *ex-vivo* quantification of MVD and MDD in GIST430 tumor sections. In addition, strong positive correlation was found between MRI and histological estimates. The current study suggests that the assessment of angiogenesis could be considered as a new promising biomarker of response to imatinib treatment. For this purpose, DCE-MRI deserves more attention at clinical level for the non-invasive assessment of treatment response by evaluating tumor vascularization properties. In view of the few therapeutic options that are currently available for imatinib-resistant patients, angiogenesis targeting may be an effective therapeutic strategy for GIST patients and functional MRI approaches may provide earlier detection of tumor response evaluation in alternative to conventional imaging modalities.

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## **Conflict of interest**

The authors have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.



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## Figure legends

**Fig.1** Implementation of GIST-T1, 882 and 430 tumor models in NSG mice. (a) Curves indicates tumor growth diameter (mm) measured by a caliber after 14, 21, 28, 35 and 42 days after bilateral, subcutaneous inoculation of GIST cell lines ( $2.5 \cdot 10^4$ ,  $2.0 \cdot 10^6$  and  $1.0 \cdot 10^6$  cells for GIST-T1, GIST882 and GIST430, respectively) in NSG mice (n=10 for each cell line). Tumors growth was detected at 21 days post inoculation. GIST-T1 and GIST430 exhibit faster kinetic growth rate in comparison to GIST882. (b) Morphological characterization of GIST tumors. *Left panel:* coronal  $T_{2w}$  MRI images acquired at 7T Bruker scanner of GIST-T1 (top), GIST882 (middle) and GIST430 (bottom) tumors (arrowhead). Presence of hemorrhagical bleedings are clearly shown in GIST-T1 tumors. *Middle panel:* representative biopsies of excised GIST-T1 (top), GIST882 (middle) and GIST430 (bottom) tumors of mice sacrificed after MRI acquisition. The arrowheads indicate the extensive bleeding in GIST-T1 tumors. *Right panel:* representative H&O staining of tumor sections belonging to GIST-T1 (top), GIST882 (middle) and GIST430 (bottom) mice acquired using an optical microscope with an objective 20 X. (c) Western blot analysis indicates increase expression of KIT receptor in GIST430 compared to GIST-T1 and GIST882. Vinculin is provided as loading control. Densitometric analysis of protein levels in at least three independent experiments is shown. Statistical analysis was performed by Student's t-test (\* $P < .05$ , \*\*  $P < .01$ )

**Fig.2** Functional MRI estimates indicate higher vessel density and permeability in GIST430 imatinib-resistant tumors, whereas GIST882 exhibit decreased cellularity. (a) Bar graphs show mean values of  $K^{trans}$  ( $\text{min}^{-1}$ , left) and  $v_p$  (right) obtained by DCE-MRI for imatinib-sensitive GIST-T1 (black) and GIST882 (grey) and imatinib-resistant GIST430 tumors (white). GIST430 tumors display significant increase of  $K^{trans}$  and  $v_p$  in comparison to GIST-T1 and GIST882. Imatinib-sensitive tumors show comparable mean values for both  $K^{trans}$  and  $v_p$ . Values are shown as mean  $\pm$  SEM. Statistical significance with: \*\* $P < .01$ ; \*\*\* $P < .001$ . (b) Representative parametric maps of  $K^{trans}$  and  $v_p$  overimposed on related  $T_{2w}$  anatomical images. GIST-T1 (left), GIST882 (middle) and GIST430 (right) show different values of  $K^{trans}$  (first line) and  $v_p$  (second line). Parametric maps highlight increased values of the pharmacokinetic parameters in GIST430 in comparison to GIST-T1 and GIST882 tumors.(c)

ADC values obtained by DW-MRI analysis. Bar graphs indicate decreased ADC ( $\text{mm}^2/\text{sec}$ ) values for GIST882 in comparison to GIST-T1 and GIST430, with statistical difference with GIST-T1. Values are shown as mean  $\pm$  SEM. Statistical significance with: \* $P < .05$

**Fig.3** *Ex-vivo* analysis confirms MRI findings by evaluating vascular density in GIST tumors. (a) Bar graph indicates microvessel density (MVD) calculated as number of vessels in GIST-T1 (black), GIST882 (grey) and GIST430 (white) tumor sections. Vessels are immunostained for the endothelial marker CD31. GIST430 tumors show increased MVD in comparison to both GIST-T1 and GIST882 tumors sections, with statistical significant difference. Significant difference is also observed between GIST-T1 and GIST882 MVD. Values are shown as mean  $\pm$  SEM. Statistical significance with: \* $P < .05$ ; \*\*\* $P < .001$ . (b) Representative immunofluorescence staining for CD31 (red) in GIST-T1 (left), GIST882 (middle) and GIST430 (right) tumor sections. Nuclei are counterstained by Hoechst (blue). Images were acquired by using a fluorescence microscopy with an objective 20 X. GIST430 section shows increased vessel density in comparison to GIST-T1 and GIST882 ones. (c) Correlation between MRI estimates  $K^{\text{trans}}$  (left) and  $v_p$  (right) and histological MVD. Strong positive correlation is observed between both MRI estimates and their related MVD values ( $v_p$ :  $P < .0001$ ,  $r = 0.82$ ;  $K^{\text{trans}}$ :  $P = .002$ ,  $r = 0.78$ )

**Fig.4** Increased vessels permeability in GIST430 tumors is confirmed by Dextran-Texas red extravasation. (a) Bar graphs indicate mean dextran density (MDD) calculated as extravasation of Dextran-Texas red in positive-CD31 immune-stained vessels. GIST430 display significant higher dextran extravasation in comparison to GIST882 and GIST-T1. Values are shown as mean  $\pm$  SEM. Statistical significance with: \*\*\* $P < .001$ . Dextran-Texas red was tail vein injected and mice were sacrificed 10 minutes later. (b) Representative images of CD31 (green), Dextran-Texas red extravasation (red) in GIST-T1 (first line), GIST882 (second line) and GIST 430 (third line) tumor sections. Images were obtained using a fluorescence microscopy with an objective 40X. Merge images are counterstained with Hoechst (blue). (c) Correlation between MRI estimates  $K^{\text{trans}}$  (left) and  $v_p$  (right) and



histological MDD. Strong positive correlation is observed between both MRI estimates and their related MDD values ( $v_p$ :  $P=.0001$ ,  $r=0.94$ ;  $K^{trans}$ :  $P=.006$ ,  $r=0.77$ ).

**Fig.5** Western Blot analysis (a) and related bar graphs (b) of VEGFR2 and VEGFR3 of GIST-T1, GIST882 and GIST430 tumor samples. GIST430 tumors reveal significantly higher expression of both receptors in comparison to GIST-T1 and GIST882 ones. Values are shown as mean  $\pm$  SEM. Statistical significance with: \* $P<.05$