

Integrin alpha-5 in human breast cancer is a mediator of bone metastasis and a therapeutic target for the treatment of osteolytic lesions

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ABSTRACT

Bone metastasis remains a major cause of mortality and morbidity in breast cancer. Therefore, there is an urgent need to better select high-risk patients in order to adapt patient's treatment and prevent bone recurrence. Here, we found that integrin alpha5 (ITGA5) was highly expressed in bone metastases, compared to lung, liver or brain metastases. High *ITGA5* expression in primary tumors correlated with the presence of disseminated tumor cells in bone marrow aspirates from early-stage breast cancer patients (n=268; $P=0.039$). *ITGA5* was also predictive of poor bone metastasis-free survival in two separate clinical datasets (n=855, HR=1.36, $P=0.018$ and n=427, HR=1.62, $P=0.024$). This prognostic value remained significant in multivariate analysis ($P=0.028$). Experimentally, *ITGA5* silencing impaired tumor cell adhesion to fibronectin, migration and survival. *ITGA5* silencing also reduced tumor cell colonization of the bone marrow and formation of osteolytic lesions *in vivo*. Conversely, *ITGA5* overexpression promoted bone metastasis. Pharmacological inhibition of ITGA5 with humanized monoclonal antibody M200 (volociximab) recapitulated inhibitory effects of *ITGA5* silencing on tumor cell functions *in vitro* and tumor cell colonization of the bone marrow *in vivo*. M200 also markedly reduced tumor outgrowth in experimental models of bone metastasis or tumorigenesis, and blunted cancer-associated bone destruction. ITGA5 was not only expressed by tumor cells but also osteoclasts. In this respect, M200 decreased human osteoclast-mediated bone resorption *in vitro*. Overall, this study identifies ITGA5 as a mediator of breast-to-bone metastasis and raises the possibility that volociximab/M200 could be repurposed for the treatment of ITGA5-positive breast cancer patients with bone metastases.

61 **INTRODUCTION**

62 Breast cancer can be successfully treated when the disease is detected early, but the patient
63 survival markedly decreases once metastatic spread occurs (1). In this respect, the prognosis for
64 patients with bone metastasis is generally poor and accompanied by skeletal complications
65 (pathological fractures, bone pain, disability) (2). Several studies have underlined that tumor cell
66 dissemination to the bone marrow is an early metastasis event and represents an independent
67 prognostic factor for poor clinical outcome (3-5). The bone marrow acts as a reservoir where
68 disseminated tumor cells (DTCs) could survive in a cell-cycle arrest state for long periods of time until
69 environmental conditions are sufficiently permissive for proliferation, at which time they become
70 competent to seed secondary organs and/or cause overt local bone metastasis (6-8). Molecular
71 mechanisms regulating bone homing and colonization by breast cancer cells remain however still
72 poorly understood.

73 In this study, we searched for potential target genes involved in breast cancer dissemination to
74 distant organs using *in silico* transcriptomic analyses of primary tumors and metastases. We found
75 that integrin alpha5 (ITGA5) is expressed at high levels in bone metastases compared to non-bone
76 metastases. Furthermore, multivariate analysis showed that *ITGA5* expression in primary breast
77 tumors is an independent prognostic factor for bone relapse. ITGA5 hetero-dimerizes with integrin
78 beta1 to form the fibronectin receptor $\alpha 5 \beta 1$ (9). In breast cancer, ITGA5 mediates tumor cell adhesion,
79 extracellular matrix-guided directional migration along fibronectin, and tumor cell survival *in vitro* (9-
80 13). ITGA5 also mediates lung metastasis in animal models of breast cancer (14,15). Additionally, a
81 synthetic peptide inhibitor derived from the synergy region of fibronectin that binds to $\alpha 5 \beta 1$ and $\alpha v \beta 3$
82 integrins (ATN-161, also called PHSCN) reduces both MDA-MB-231 breast cancer bone metastasis
83 formation and skeletal tumor outgrowth (14,16). However, ATN-161 interacts with $\alpha v \beta 3$ (16), and the
84 treatment of tumor-bearing animals with a specific nonpeptide antagonist of $\alpha v \beta 3$ (PSK 1404) also
85 inhibits bone metastasis formation (17), suggesting that the inhibitory effect of ATN-161 on bone
86 metastasis formation was mediated through the therapeutic targeting of $\alpha v \beta 3$. Beside ATN-161, a

87 humanized IgG4 monoclonal antibody against $\alpha 5\beta 1$, known as M200 (volociximab), was developed as
88 an antiangiogenic agent for the treatment of solid tumors and age-related macular degeneration
89 (18,19). A phase-I study conducted in 22 patients with advanced stage solid tumors showed that the
90 pharmaco-toxicologic profile of M200 is safe, and preliminary evidence of antitumor activity was
91 reported in one patient with renal cell carcinoma (18). Clinical trials also evaluated its safety in the
92 treatment of ovarian cancer and non-small cell lung cancer, as a single agent or in combination with
93 chemotherapy (20,21).

94 Here, we provide evidence that ITGA5 is a mediator of bone metastasis and a potential
95 therapeutic target for bone metastasis treatment. Using genetic overexpression or silencing strategies,
96 we show that ITGA5 in breast cancer cells mediates metastatic tumor cell colonization of the bone
97 marrow and promotes formation of osteolytic lesions *in vivo*. Furthermore, we show that M200 could
98 be effective in the treatment of breast cancer patients with osteolytic bone metastases by targeting
99 both tumor cells and osteoclasts, the latter being bone-resorbing cells that mediate cancer-induced
100 bone destruction.

101

102 RESULTS

103 ITGA5 is a bone metastasis-associated gene in breast cancer

104 We compared the transcriptomic profile of 21 bone metastases with that of 59 metastases from
105 other distant organs. This analysis identified 246 genes (gene set #1) that were expressed at higher
106 levels in bone metastases compared to non-bone metastases (**Fig. 1a** and **Table S1**). In parallel, the
107 analysis of 855 radically resected primary breast tumors with known location of the first distant
108 metastasis led to 146 genes (gene set #2) that were significantly upregulated in primary tumors from
109 patients who first relapsed in bone, compared to patients who first relapsed at non-bone metastatic
110 sites or did not relapse after 200 months follow-up (**Fig. 1b** and **Table S1**). Nine genes were common
111 to gene sets #1 and #2: *EFEMP2* (EGF-containing fibulin-like extracellular matrix protein 2), *ITGA5*
112 (integrin alpha 5), *KIAA1199* [cell migration-inducing and hyaluronan-binding protein (CEMIP)],
113 *MFAP5* (microfibrillar-associated protein 5), *PLXDC1* (plexin domain-containing protein 1), *SPOCK1*
114 [SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 1], *TCIRG1* (T Cell Immune
115 Regulator 1) and *TGFB111* (Transforming Growth Factor Beta 1 Induced Transcript 1) (**Fig. 1c**).
116 Beside the role played by *ITGA5* in promoting breast cancer cell adhesion, invasion and survival (9-
117 16), *EFEMP2*, *KIAA1199*, and *MFAP5* also enhance breast cancer motility and invasiveness (22-24).
118 *SPOCK1* and *TGFB111* [also called hydrogen peroxide-inducible clone 5 (Hic-5)] are induced by TGF-
119 β and promote breast cancer cell invasion (25,26). *PLXDC1* increases invasion in gastric cancer (27),
120 and *TCIRG1* is an osteoclast-specific vacuolar proton pump subunit that acts as a metastasis
121 enhancer in hepatocellular carcinoma (28). In addition, *MFAP5* is upregulated in human breast cancer
122 bone metastases compared to primary tumors (24).

123 The functional importance of these genes was assessed by gene network analysis, revealing a
124 prominent role for *ITGA5*, given its high connectivity degree within the network structure (**Fig. S1**).
125 Moreover, as shown in **Fig. 1d**, *ITGA5* was highly expressed in bone metastases compared to lung (P
126 $= 0.001$), liver ($P = 5.10^{-5}$) and brain ($P = 3.10^{-4}$) metastases. We therefore focused our attention to the
127 role of *ITGA5* in breast cancer bone metastasis.

128 **ITGA5 is an independent prognostic factor for breast cancer bone metastasis**

129 We quantified *ITGA5* expression levels in 427 radically resected primary breast tumors (29).
130 Kaplan-Meier survival analysis revealed that the risk of bone metastasis was significantly higher for
131 patients with high *ITGA5* levels (HR= 1.62, $P = 0.024$) (**Fig. 1e**). Furthermore, *ITGA5* predicted bone
132 relapse ($P = 0.028$) independently of clinicopathological characteristics (**Table 1**). To confirm these
133 findings, we conducted *in silico* analysis of a cohort of 855 radically resected primary mammary
134 tumors with clinical annotation for recurrences and observed that breast cancer patients with tumors
135 expressing high *ITGA5* mRNA levels were more likely to relapse in bone (HR= 1.36, $P = 0.018$) (**Fig.**
136 **S2**). After adjusting for clinicopathological factors, *ITGA5* remained significantly associated with bone
137 relapse ($P = 0.034$) (**Fig. S2**).

138

139 **Elevated ITGA5 protein levels in primary tumors are associated with the presence of DTCs in**
140 **bone marrow aspirates from patients with breast cancer**

141 To examine the potential contribution of *ITGA5* in the homing of breast cancer cells to bone,
142 we analyzed by immunohistochemistry *ITGA5* protein levels in 268 radically resected primary tumors
143 from a cohort of breast cancer patients with no clinical signs of metastasis for whom the presence or
144 absence of DTCs in the bone marrow was documented (**Table S2**) (30). A significantly higher
145 percentage of breast cancer patients having elevated *ITGA5* protein levels in primary tumors were
146 DTC-positive ($P = 0.039$), compared to that observed for patients with low *ITGA5* levels in primary
147 tumors (**Fig. 1f and Table S2**). Additionally, flow cytometry analysis of a breast cancer DTC cell line
148 (BC-M1) (31,32) showed cell surface expression of integrin $\alpha 5 \beta 1$ (**Fig. S3A**).

149

150 **ITGA5 promotes breast cancer cell dissemination to the bone marrow and formation of**
151 **osteolytic bone metastases *in vivo***

152 Human MDA-MB-231, Hs578T and MDA-B02 breast cancer cells, which are ER- and PR-
153 negative and do not bear an amplification of HER-2 gene (referred to as triple-negative breast cancer

154 cells), had higher cell surface expression levels of integrin $\alpha 5 \beta 1$ and higher ITGA5 protein levels than
155 luminal A (T47D, MCF-7, BT-474) and HER2-expressing luminal B (SKBr3) breast cancer cell lines, as
156 judged by flow cytometry and western blotting, respectively (**Fig. S3A, B**). *ITGA5* mRNA expression
157 levels in tumor cells were further investigated using 51 distinct breast cancer cell lines with different
158 molecular phenotypes and degree of invasiveness (GSE12777) (33). A significant correlation was
159 observed between high *ITGA5* mRNA expression levels and high tumor cell invasiveness ($P = 0.0083$)
160 (**Fig. S3C, D**). In particular, the highest *ITGA5* mRNA levels were observed in claudin-low, triple-
161 negative breast cancer cell lines ($P < 0.01$) (**Fig. S3E**).

162 We therefore silenced ITGA5 in claudin-low MDA-MB-231 and MDA-B02 breast cancer cells,
163 the latter being a bone metastatic cell subpopulation of the MDA-MB-231 cell line, which constitutively
164 and specifically overexpresses $\alpha \nu \beta 3$ integrin compared to the parental cell line (17, 34). ShRNA-
165 mediated silencing of *ITGA5* in these cells drastically reduced ITGA5 expression, both at the protein
166 and cell surface expression levels compared to shRNA control cells (**Fig. 2a, b**). The flow cytometry
167 analysis of shITGA5-MDA-MB-231 and shITGA5-MDA-B02 cells showed that the silencing of ITGA5
168 did not modify cell surface expression levels of integrin subunits $\alpha 2$, $\alpha 3$, $\alpha 4$ and $\beta 1$ and of $\alpha \nu \beta 3$
169 integrin, when compared to shRNA control cells (**Figs. S4 and S5**). The silencing of ITGA5 led to a
170 60% reduction of tumor cell adhesion to fibronectin (**Fig. S6A**), whereas tumor cell adhesion to glass,
171 poly-D-Lysine and laminin remained unchanged (**Fig. S6B**). In addition, *ITGA5* silencing reduced by
172 half the number and size of mammospheres formed by MDA-B02-shITGA5 cells, compared to that
173 observed with MDA-B02-shCtrl cells (**Fig. S6C**).

174 To investigate whether ITGA5 could drive tumor cell anchorage in bone marrow *in vivo*, MDA-
175 MB-231 cells that have the propensity to form lung and bone metastases were injected into the tail
176 artery of immunodeficient mice and, two weeks after tumor cell inoculation, these animals were culled
177 and the number of micrometastases in bone marrow and lungs quantified. Abrogating *ITGA5*
178 expression in MDA-MB-231-ShITGA5 cells significantly reduced bone marrow micrometastasis
179 formation ($P = 0.015$), whereas the extent of tumor cell dissemination to lungs remained unchanged,

180 compared to MDA-MB-231-Sh-Ctrl cells (**Fig. 2c**). These results may be explained by the fact
181 fibronectin was strongly expressed in bone tissue, whereas only a weak expression was observed in
182 lungs, the immunostaining being essentially localized around blood vessels within the lung
183 parenchyma (**Fig. 2d**). Moreover, high fibronectin expression levels in bone marrow stroma were
184 observed compared to lung parenchyma, when analyzing EST profiles of *Mus Musculus* and *Homo*
185 *Sapiens* tissue samples (**Fig. S7**). Thus, these data suggested that ITGA5 preferentially mediates
186 tumor cell anchorage in the bone marrow by binding to fibronectin.

187 To determine whether ITGA5 could play a role in the formation and progression of bone
188 metastases, bone-seeking MDA-B02 cells, silenced or not silenced for *ITGA5*, were injected into the
189 tail artery of immunodeficient mice. Radiographic analysis of tumor-bearing animals 4 weeks after
190 tumor cell inoculation showed a significant decrease ($P = 0.0268$) of the extent of osteolytic lesions in
191 hind limbs of mice injected with MDA-B02-shITGA5 cells compared to that observed with MDA-B02-
192 shCtrl cells (**Fig. S8**).

193 Experiments were also conducted with human MCF-7 breast cancer cells that express low
194 amounts of ITGA5 (**Fig. S3A, B**). Transduction of luciferase2-expressing MCF-7 cells (MCF-7-luc2)
195 with a retroviral plasmid containing the ITGA5 open reading frame (MCF-7-luc2-ITGA5) resulted in a
196 strong expression of integrin $\alpha 5 \beta 1$ (**Fig. 3a, b**). As judged by flow cytometry analysis, ITGA5
197 overexpression led to decreased cell surface expression levels of $\alpha 2$ and $\alpha 3$ integrins, whereas cell
198 surface expression levels of $\alpha 4$, $\beta 1$ and $\alpha v \beta 3$ remained unchanged, when compared to control MCF-7-
199 luc2 cells (**Figs. S4 and S5**). Integrin $\alpha 2 \beta 1$ and $\alpha 3 \beta 1$ are acting as cell surface receptors for collagen
200 and laminin, respectively (35). We cannot exclude a decreased attachment of MCF-7-luc2-ITGA5 cells
201 to these extracellular matrix proteins. However, as expected, MCF-7-luc2-ITGA5 cell adhesion and
202 spreading to fibronectin was increased compared to that observed with MCF-7-luc2-Ctrl cells (**Fig.**
203 **3c**). MCF-7-luc2-ITGA5 or MCF-7-luc2-Ctrl cells were therefore inoculated intra-arterially to *nude* mice
204 (**Fig. 3d, e**). Bioluminescence imaging revealed an earlier onset ($P = 0.0186$) of skeletal tumor burden
205 in mice injected with MCF-7-luc2-ITGA5 cells, compared to animals bearing MCF-7-luc2-Ctrl cells

(**Fig. 3d**). Microcomputed tomography of metastatic long bones showed that the BV/TV ratio (a measure of the bone volume) was decreased ($P = 0.035$) in mice inoculated with MCF-luc2-ITGA5 cells, indicating a higher extent of bone destruction compared to animals bearing MCF-7-luc2-Ctrl tumor cells (**Fig. 3e**).

Overall, these data indicated that ITGA5 mediates the homing of breast cancer cells in the bone marrow and promotes formation of osteolytic bone metastases *in vivo*.

Pharmacological inhibition of ITGA5 reduces breast cancer cell dissemination to the bone marrow and formation of osteolytic bone metastases *in vivo*

We examined the therapeutic potential of targeting ITGA5 for the treatment of bone metastasis, using a humanized monoclonal antibody against $\alpha 5\beta 1$ (M200, volociximab) (18). Antibody M200 selectively binds to human $\alpha 5\beta 1$, but not murine $\alpha 5\beta 1$ (18). *In vitro*, M200 treatment dose-dependently decreased MDA-B02 cell adhesion to fibronectin ($P < 0.0003$), but not to type I collagen, or vitronectin (**Fig. 4a**). The paxillin immunofluorescent labelling of focal adhesion contacts showed that M200 specifically inhibited MDA-B02 and MDA-MB-231 cell spreading to a fibronectin matrix, but not to type I collagen or vitronectin (**Fig. 4b**). In line with this inhibitory effect on tumor cell spreading, M200 treatment dose-dependently reduced MDA-B02 cell migration through inserts coated with fibronectin ($P < 0.001$) (**Fig. 4c**). *In vivo*, immunodeficient mice were treated with M200 or a negative control IgG antibody beginning 1 day (D-1) before intra-arterial inoculation of MDA-B02 cells (D0). The treatment with the antibody then continued every other day until day 7 (D7), at which time animals were culled, and the bone marrow collected and placed under antibiotic selection, enabling the selective outgrowth of antibiotic-resistant tumor cells (**Fig. 4d**). After two weeks in culture, the average number of tumor cell colonies recovered in the bone marrow from animals treated with M200 was significantly impaired, compared to that recovered from animals treated with a control IgG (7 ± 2 and 128 ± 10 colonies/well, respectively; $P < 0.0003$) (**Fig. 4d**). Using a similar treatment protocol from D-1 to D28 (**Fig. 5a**), antibody M200 also significantly delayed the onset of skeletal tumor burden and extent of osteolytic

lesions in animals (**Fig. 5b, c**). Histomorphometric analysis of metastatic legs from M200-treated animals showed that the BV/TV ratio was enhanced when compared with control IgG-treated tumor-bearing animals (**Fig. 5d**). This difference was accompanied by a sharp reduction in the TB/STV ratio (a measure of the skeletal tumor burden) (**Fig. 5d**). Moreover, immunostaining of metastatic legs showed a concomitant decrease of Ki-67 index, a measure of tumor cell proliferation (M200, 15.5 ± 2.5 % vs Ctrl IgG, 28.5 ± 2.5 %; $P < 0.05$) (**Fig. 5d**).

To be free from the impact of bone-derived growth factors released from resorbed bone that stimulate tumor growth, the anti-tumor potential of antibody M200 was investigated in animals bearing subcutaneous MDA-B02 tumor xenografts (**Fig. 6a**). A statistically significant reduction of tumor growth was observed in M200-treated tumor-bearing animals, compared to control IgG-treated tumor-bearing animals ($P < 0.005$) (**Fig. 6b, c**). At day 35 after tumor cell inoculation, the median weight of tumors from M200-treated animals was almost 3-fold lower than that of tumors from Ctrl IgG-treated animals ($P = 0.05$) (**Fig. 6d**). Similarly, ITGA5 silencing in MDA-B02 cells substantially reduced subcutaneous growth of MDA-B02-shITGA5 tumors compared to control (**Fig. S9**). Furthermore, cell cycle analysis showed that M200 treatment inhibited MDA-B02 cells entering into S phase, when compared to that observed with a negative control IgG antibody (**Fig. S10**). Thus, M200 exhibits a direct anti-tumor effect *in vitro* and *in vivo*.

Anti-ITGA5 function-blocking antibody M200 decreases human osteoclast differentiation and activity.

M200 did not interfere with murine osteoclastogenesis induced by RANKL and MCS-F in combination with the conditioned medium from MDA-B02 cells (**Fig. S11A**), nor did it modulate gene expression of murine osteoblast during osteoblastogenesis *in vitro* (**Fig. S11B**). However, ITGA5 is expressed in human osteoclasts (36). We therefore tested the effect of M200 on human osteoclasts, using PBMCs treated with RANKL and MCS-F to induce osteoclast differentiation, as previously

258 described (37). When compared to cathepsin K (an osteoclast marker), osteoclasts did express
259 ITGA5, both at the mRNA and protein levels (**Fig. 7a, b**). ITGA5 expression gradually decreased
260 during the course of osteoclast differentiation (**Fig. 7b, c**). Nevertheless, M200 was nearly as potent
261 as anti-RANKL antibody denosumab to inhibit human osteoclast differentiation *in vitro*, compared to a
262 control IgG (**Fig. 7d**). Furthermore, antibody M200 inhibited osteoclast activity, decreasing by 80% the
263 resorption of a synthetic inorganic bone matrix (**Fig. 7e**). By contrast, it did not affect osteoclast
264 viability (**Fig. 7f**).
265

DISCUSSION

Our study establishes a bone-metastasis-promoting role for *ITGA5* in breast cancer. Specifically, we report that high *ITGA5* levels in primary tumors were predictive of poor bone metastasis-free survival in two separate clinical datasets (HR=1.36, $P=0.018$ and HR=1.62, $P=0.024$). Additionally, using a clinical cohort of breast cancer patients without any clinical signs of metastasis, we showed that high *ITGA5* expression levels in primary tumors correlated with the presence of DTCs in the bone marrow. Moreover, *ITGA5* was expressed in human DTCs (this study and 12). Our clinical data are consistent with those obtained in previous prospective clinical trials demonstrating that the risk of recurrence in early-stage breast cancer is significantly higher in patients with detectable DTCs in the bone marrow than in those without (3-5). These findings (3-5,12 and this study) collectively suggest that *ITGA5* mediates DTC colonization of the bone marrow. This contention was also supported by our preclinical data. Using genetic silencing and overexpression strategies or pharmacological inhibition, we uncovered a specific association between *ITGA5* expression levels in breast cancer and the development of bone metastasis. Although bone is a predominant site of metastasis for ER-positive breast cancer with a frequency as high as 65 to 70%, triple negative breast cancer exhibits a rate of bone metastasis (39%) similar to lung metastasis (43%), which is comparatively higher than that observed in other distant metastatic sites such as brain (25%) and liver (21%) (38). *ITGA5* could therefore contribute to the tropism of triple negative breast cancer cells to bone. Several factors have been shown to regulate *ITGA5* expression in triple negative breast cancer (10, 39-41). For example, steroid receptor coactivator (SRC-1), which is an ER transcriptional coactivator, enhances *ITGA5* expression in ER-negative breast cancer cells (10). Additional factors expressed by human breast cancer cells, such as PTH-rP and angiopoietin-2, promote tumor cell adhesiveness to fibronectin and tumor cell motility and invasion through the specific up-regulation of *ITGA5* (39,40). Conversely, members of the miR-30 family impede breast cancer bone metastasis formation by directly targeting *ITGA5* (41). In this respect, *ITGA5* silencing in Hs578T cells (a triple-negative breast cancer cell line expressing high *ITGA5* levels) recapitulates inhibitory effects of miR-

30s on bone metastasis formation *in vivo* (41). Here, we found that abrogating *ITGA5* in human MDA-MB-231 cells also blunted tumor burden in the bone marrow, whereas the formation of pulmonary micrometastases remained unaffected. These results may be explained by the fact that fibronectin is naturally expressed in the bone stroma, whereas its expression in lung parenchyma is essentially localized around blood vessels (11,42 and this study). This observation does not preclude a role for *ITGA5* in lung metastasis. Indeed, *ITGA5* has been associated with lung metastasis in animal models of breast cancer (14,15). However, our study suggests that additional molecular mechanisms associated with lung metastasis formation may likely compensate for the lack of *ITGA5* in human breast cancer cells, whereas *ITGA5* is crucial for the homing of these cancer cells in the bone marrow. It has previously been reported that *ITGA5* promotes survival of breast cancer cells in the bone marrow (12). Here, we showed that *ITGA5* silencing reduced the survival of breast cancer cells (**Fig. S6C**). We therefore propose that *ITGA5* provides breast cancer cells (and DTCs) with a survival advantage by binding to fibronectin in the bone marrow, which explains, at least in part, why high *ITGA5* expression levels in primary tumors predict the occurrence of future bone metastases in patients with early-stage breast cancer.

We previously reported that bone-seeking MDA-B02 cells specifically overexpress $\alpha\beta3$ integrin, compared to parental MDA-MB-231 cells (34 and **Fig. S5**), and its overexpression by MDA-MB-231 cells reproduces the bone metastatic phenotype of MDA-B02 cells *in vivo* (17). Here, *ITGA5* had a bone-metastasis-promoting role, whereas *ITGA5* expression in MDA-B02 cells was 20% lower than that observed in MDA-MB-231 cells (**Fig. S4**). Although counterintuitive, expression levels of integrins are not always a direct readout of integrin functions in cells (35). Additional levels of regulation exist. Upon binding of integrins to extracellular matrix proteins, there is a crosstalk between integrins that determines downstream signaling and cell behavior (35). For example, $\alpha5\beta1$ and $\alpha\beta3$ integrins both bind fibronectin and this is the collaborative interactions among these two integrins rather than their respective expression levels that determine cell migration response toward fibronectin (43). Interestingly, the knockdown of integrin $\beta1$ in murine 4T1 triple negative breast cancer cells

318 induces a compensatory increase in $\beta 3$ integrin expression and a switch in the migratory behavior of
319 4T1 cells from collective to single cell movement *in vitro* that leads to metastasis *in vivo* (44). The $\beta 1$
320 integrin subunit heterodimerizes with different α subunits (35), which probably explains why ITGA5
321 silencing did not modify cell surface expression levels of $\beta 1$ and $\alpha v \beta 3$ in MDA-MB-231 and MDA-B02
322 cells (**Figs. S4 and S5**). Yet, it is conceivable that $\alpha 5 \beta 1$ helps triple negative breast cancer cells
323 survive in the bone marrow until environmental conditions are sufficiently permissive for tumor growth,
324 at which time integrin switching from $\alpha 5 \beta 1$ to $\alpha v \beta 3$ triggers pro-invasive signals. This hypothesis
325 warrants further investigation.

326 Having shown that there is an explicit role for ITGA5 in mediating early breast cancer cell
327 colonization in the bone marrow, we then investigated whether ITGA5 also plays a role in the
328 development of metastatic skeletal lesions. In bone metastasis, there is a vicious cycle where tumor
329 cells stimulate osteoclast-mediated bone resorption and, in turn, bone-derived growth factors released
330 from resorbed bone stimulate skeletal tumor burden (2). We showed that the silencing or
331 pharmacological inhibition of ITGA5 markedly reduced tumor outgrowth in experimental models of
332 bone metastasis or tumorigenesis. This inhibition of tumor growth (and subsequent overall decrease in
333 the secretion of tumor-derived pro-osteoclastic factors) led to the reduction in osteoclast-mediated
334 bone destruction as would be predicted by the vicious cycle theory. However, there is some evidence
335 in the literature showing that human osteoblasts and osteoclasts do express ITGA5 (36,45). It is
336 possible that M200 could act upon human osteoblast differentiation. Here, we found that a clinically
337 relevant concentration of M200 antibody was nearly as effective as the anti-RANKL antibody
338 denosumab to inhibit human osteoclast differentiation and activity *in vitro*. Thus, in addition to its anti-
339 tumor effect, we anticipate antibody M200 may also be effective at inhibiting bone resorption.

340 Clinical trials have repeatedly failed to demonstrate therapeutic benefits of integrin inhibitors in
341 cancer patients (35). Volociximab has shown preliminary evidence of efficacy in early phase I/II trials
342 but failed in larger phase III trials (18,20,21). However, none of these clinical trials using patients with
343 advanced cancer and metastasis have specifically addressed the efficacy of volociximab on bone

344 metastasis. A focus for further work would be to establish if ITGA5-positive breast cancer patients with
345 bone metastases are likely to benefit from volociximab in combination with denosumab, which is the
346 best standard of care for prevention of the skeletal morbidity associated with bone metastases in
347 patients with advanced malignancies (2).

348

349 **MATERIAL AND METHODS**

350 **Patients**

351 *ITGA5* mRNA expression was quantified by RT-qPCR in primary breast carcinomas obtained from the
352 Curie Institute/René Huguenin Hospital (Saint-Cloud, France) (29). *ITGA5* protein
353 immunohistochemistry was performed using primary tumors from breast cancer patients for whom the
354 presence or absence of DTCs in bone marrow aspirates was known (University Medical Center
355 Hamburg-Eppendorf, Germany) (30,31,46,47).

356

357 **Analysis of human breast tumor microarray data sets**

358 Analysis were conducted using public breast cancer microarray data sets GSE2034, GSE12276,
359 GSE2603 and NKI295, consisting of 855 patients with clinical outcomes (48), and data sets
360 GSE11078 and GSE14020 for 80 distant breast cancer metastases (49,50).

361

362 **Real-Time qPCR**

363 PCR experiments were conducted as previously described (51). All primers are shown in **Table S3**.

364

365 **Tissue microarray and immunohistochemistry**

366 Immunodetection of *ITGA5* in breast tumor tissue microarrays was performed following a previously
367 described method (4).

368

369 **Cell lines and cell transduction**

370 Human breast cancer cell lines T47D, MCF-7, Hs587T, SKBR3, BT-474 and MDA-MB-231 were
371 obtained from the American Type Culture Collection (ATCC, Manassas, VA) and authenticated using
372 short tandem repeat analysis. The human MDA-B02 breast cancer cell line (MDA-B02) is a

373 subpopulation of the MDA-MB-231 cell line (MDA-MB-231) that was selected for its high and selective
374 efficiency to metastasize to bone in mice (34,52).

375

376 Stable silencing of ITGA5 was achieved in luciferase-2-expressing MDA-MB-231 and MDA-BO2 cells
377 (MDA-231-shITGA5 and MDA-BO2-shITGA5, respectively) by transduction with lentiviral plasmids
378 containing hairpin shRNAs targeting ITGA5. *ITGA5* was overexpressed in luciferase-2-expressing
379 MCF-7 cells (MCF-7luc2 ITGA5) using the amphotropic retroviral packaging system (Clontech).

380

381 **Cell-based assays**

382 Tumor cell functions were investigated using cell adhesion and migration assays, and cell cycle
383 analysis, as previously described (41,53,54). The effect of antibody M200 on differentiation of mature
384 osteoclasts or osteoblasts was studied using previously described methods (37,41,55).

385

386 **Animal studies**

387 All procedures involving animals, including housing and care, method of euthanasia, and experimental
388 protocols were conducted in accordance with a code of practice established by the local ethical
389 committee (Comité d'Expérimentation Animale de l'Université Claude Bernard Lyon 1, CEEA-55)
390 under project licence MESR number: APAFIS#4798-2016040510106615. Four-week-old female
391 BALB/c nude mice were purchased from Janvier Laboratories (Saint-Berthevin). For bone metastasis
392 experiments, immunodeficient BALB/c female *nude* mice were randomly assigned to receive
393 intraperitoneal injection of M200 antibody or control IgG (15 mg/kg) one day before tumor cell
394 injection. MDA-BO2 cells were then inoculated into the tail artery ($5 \cdot 10^5$ /100 μ L of PBS) of
395 anesthetized mice at day 0. Alternatively, MDA-BO2 shCtrl or MDA-BO2 shITGA5 cells ($5 \cdot 10^5$ /100 μ L
396 of PBS) were inoculated into the tail artery. Hormone-responsive MCF-7luc2 ITGA5 and MCF-7luc2
397 control cells (4×10^5 cells /100 μ L of PBS) were injected intra-arterially to female BALB/c *nude* mice

two days after subcutaneous implantation of 17 β -estradiol pellet in animals. Measurements of the extent of osteolytic lesions and skeletal tumor burden were performed by radiography/micro-computed tomography and bioluminescence imaging, respectively, as previously described (41,52-54). On day 28 after tumor cell inoculation, anesthetized mice were sacrificed by cervical dislocation and hind limbs were collected and embedded in paraffin for further analyses by histomorphometry, histology and immunohistochemistry. *Ex vivo* bone marrow micrometastasis and tumorigenesis experiments were conducted as previously described (41,52-54).

405

406 **Statistical analysis**

All statistical analyses were performed using PASW Statistics (version 21.0; SPSS Inc, Chicago) or GraphPad Prism (version 5, San Diego). All *in vitro* experimental procedures consisted of at least 3 independent biological repeats, and appropriate negative and positive controls. Comparisons were performed using two-sided unpaired Student-t test or ANOVA test followed by Tukey's test for *in vitro* experiments and by Mann-Whitney U test for *in vivo* experiments with significance at <0.05 being used to determine significant differences. Cox proportional regression model was used to estimate hazard ratios and 95% CIs for distant relapse to bone in relation to the ITGA5 expression as a continuous variable, with adjustment for classic prognostic factors: age, tumor size, node involvement, estrogen receptor and progesterone receptor status, and Her2 status. Survival analyses were visualized using Kaplan-Meier plots and differences in survival across the strata were calculated using a log-rank *P* test. In total, data were obtained from 855 patients with, as the first site of relapse, bone (n=238), brain (n=49), lung (n=101) or liver metastasis (n=107). For analyses of bone relapse free survival, non-bone events, including liver, lung and brain relapses were censored.

420

The power calculation for *ex vivo* experiments is based on our previous work (41,53,54), showing that bone marrow micrometastases occur in 80% of animals. Similarly, for bone metastasis experiments,

423 80% of animals have skeletal lesions 4 weeks after intra-arterial tumor cell inoculation (17,41,52-54).
424 Assuming a power of 80% and a level of significance of 5%, we estimated that we will be able to
425 measure a difference of 60% or greater with 10 animals per group, using a Mann-Whitney test. With
426 regard to tumor xenograft experiments, the tumor take in this animal model is 90-100%. From our
427 previous work (17,41,52-54), assuming a power of 80% and a level of significance of 5%, we
428 estimated that we will be able to measure a difference of 60% or greater with 5 animals per group,
429 using a Mann-Whitney test. Only the animals that were alive at the end of the protocols were included
430 in the statistical analyses.

431

432 In order to avoid bias for *in vivo* experiments, staff injecting transduced tumor cells into animals were
433 different from those assessing the effects of transduction. Mice and subsequent tissue samples were
434 labelled such that staff assessing the effects of transduction and analyzing the results were unaware
435 which group received mock-transduced tumor cells or tumor cells in which ITGA5 was silenced or
436 overexpressed until analyses were complete.

437

438 **Additional methods**

439 A more detailed description of methods outlined above, and additional methods used in this study are
440 provided in the Supplementary Methods section.

441

442

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445 (Department of Gynecology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany). We
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447 in imaging and flow cytometry.

448

449 **COMPETING INTERESTS**

450 The authors declare no conflicts of interest.

451

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- 599

600 **FIGURE LEGENDS**

601 **Figure 1:** ITGA5 is a bone metastasis-associated gene in breast cancer. **(A)** Heat map analysis of
602 genes that are highly expressed in bone metastases (n=21) compared to visceral metastases (n=59).
603 Each row represents a gene, and each column represents a metastasis specimen. Class comparison
604 analysis was performed using a univariate *t* test ($p < 10^{-4}$, fold-change > 1.5). **(B)** Heat map of genes
605 associated with bone metastasis-free survival. Transcriptomic gene profile of 855 radically resected
606 primary breast tumors were hierarchized according to the location of the first distant metastasis. *Bone*
607 *metastasis-negative patients*: patients who first relapsed at non-bone sites or did not relapse after 200
608 months follow-up. *Bone-metastasis-positive patients*: patients who first relapsed in bone after 100
609 months follow-up. **(C)** Venn diagram of (a) genes upregulated in bone *versus* visceral breast cancer
610 metastasis (gene set #1) and (b) genes upregulated in primary tumors from patients with early
611 occurrence of bone metastasis (gene set #2), which underscores a set of 9 genes (gene set #3)
612 shared between gene sets #1 and #2. *EFEMP2*: EGF-containing fibulin-like extracellular matrix protein
613 2. *ITGA5*: integrin alpha 5. *KIAA1199*: Cell migration-inducing and hyaluronan-binding protein
614 (CEMIP). *MFAP5*: Microfibrillar-associated protein 5. *PLXDC1*: Plexin domain-containing protein 1.
615 *SPOCK1*: SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 1. *TCIRG1*: T Cell
616 Immune Regulator 1. *TGFB111*: Transforming Growth Factor Beta 1 Induced Transcript 1. **(D)** *ITGA5*
617 mRNA expression levels in breast cancer metastases. Data are expressed as mean \pm SEM. **(E)**
618 Kaplan-Meier estimates for rates of bone metastasis-free survival of breast cancer patients (n = 427),
619 according to high and low *ITGA5* expression levels. HR: hazard ratio; CI: confident interval. HR and
620 95% CI are based on Cox univariate analysis. **(F)** Percentage of breast cancer patients with DTCs in
621 the bone marrow according to high or low *ITGA5* expression levels in matched primary mammary
622 tumors (n = 268). *ITGA5+*: *ITGA5*-high. *ITGA5-*: *ITGA5*-low. *: $P = 0.039$.

623

624 **Figure 2:** ITGA5 in triple negative MDA-MB-231 and MDA-B02 breast cancer cells promotes the
625 burden of micrometastatic disease in the bone marrow *in vivo*. **(A)** *ITGA5* expression in MDA-MB-231

626 and MDA-B02 cells silenced for ITGA5 (shITGA5), compared to control cell lines (shCtrl), as
 627 measured by western blotting. **(B)** Cell surface expression levels of integrin $\alpha 5\beta 1$ in MDA-MB-231-
 628 (left-hand panel) and MDA-B02-shCtrl and shITGA5 cells (right-hand panel), as measured by flow
 629 cytometry using anti-ITGA5 monoclonal antibody IIA1 (red and black histograms) or an isotype-
 630 matched negative control antibody (grey histograms). **(C)** *Top-left panel:* Schematic representation of
 631 the experimental protocol. MDA-MB-231-shCtrl or MDA-MB-231-shITGA5 cells were inoculated intra-
 632 arterially to Balb/c *nude* mice (n = 5 per group). Two weeks after tumor cell inoculation, animals were
 633 culled, and the bone marrow and lungs collected for tumor cell colony assays. *Bottom-left panel:*
 634 Representative images of tumor cell colonies in the bone marrow and lung are shown for each cell
 635 line. *Right panel:* Bar graphs showing the average number of tumor cell colonies formed in the bone
 636 marrow and lungs for each cell line. Data are expressed as the mean \pm SEM. **(D)** Fibronectin
 637 immunostaining in bone and lung. Right-hand panels are a magnification of insets shown in left-hand
 638 panels. In bone, a strong immunostaining for fibronectin was observed in osteoblasts (white arrows),
 639 osteocytes (red asterisks) and endothelial cells (black arrows). In lungs, the immunostaining was
 640 mainly localized around blood vessels.

641

642 **Figure 3:** Overexpression of ITGA5 in human luminal-A MCF-7 breast cancer cells promotes tumor
 643 cell adhesion to fibronectin *in vitro* and enhances skeletal tumor burden and the extent of metastatic
 644 osteolytic lesions *in vivo*. **(A)** Western blot analysis of ITGA5 in MCF-7-luc2 cells (MCF-7 Ctrl) after
 645 transduction with the retroviral plasmid (MCF-7 ITGA5). **(B)** Flow cytometry analysis of cell surface
 646 expression of $\alpha 5\beta 1$ integrin in MCF-7-Ctrl and MCF-7-ITGA5 cells. **(C)** *Left panels:* Representative
 647 images of MCF-7-Ctrl and MCF-7-ITGA5 cell adhesion to fibronectin as a function of time. *Right panel:*
 648 Quantification of the number of adherent cells to fibronectin at 20 and 40 min. *: $P < 0.001$, **: P
 649 < 0.0001 . **(D)** *Top panel:* Schematic representation of the experimental protocol. MCF7-luc2-Ctrl or
 650 MCF7-luc2-ITGA5 cells were inoculated intra-arterially to Balb/c *nude* mice (n = 4 to 5 per group).
 651 Eighty days after tumor cell inoculation, animals were analyzed by bioluminescence imaging and

652 histomorphometry of metastatic legs was measured by microcomputed tomography (μ CT). *Bottom-left*
653 *panel*: whole-body bioluminescence imaging of a representative animal for each group at day 80 after
654 tumor cell inoculation. *Bottom-right panel*: Kaplan-Meier estimates for rates of invasive-disease-free
655 survival (IDFS) of animals, as measured by bioluminescence imaging. **(E)** *Left panel*: μ CT of
656 representative metastatic tibiae for each group. *Right panel*: Assessment of bone destruction by
657 histomorphometry, as measured by the bone volume (BV)/tissue volume (TV) ratio of metastatic legs
658 from mice injected with MCF7-luc2-Ctrl (n=5) or MCF7-luc2-ITGA5 cells (n=4).

659

660 **Figure 4:** Function-blocking humanized anti-ITGA5 monoclonal antibody M200 specifically inhibits
661 breast cancer cell adhesion and spreading to fibronectin and blocks MDA-B02 cell colonization in the
662 bone marrow. **(A)** MDA-B02 cells treated or not treated with increasing concentrations of M200 (50,
663 150 and 250 μ g/ml) were allowed to adhere for 1 hour to human fibronectin, type I collagen or
664 vitronectin. Attached cells were then fixed, stained and counted under microscope. Data are
665 expressed as mean \pm SEM of three separate experiments. ***: $P < 0.0001$. **(B)** Representative
666 images of paxillin immunofluorescent labelling of focal adhesion contacts (black spots at the edge of
667 the plasma membrane) in MDA-MB-231 and MDA-B02 cells treated with a control IgG or M200 (250
668 μ g/ml) that attached and spread to fibronectin, type I collagen or vitronectin. **(C)** Effect of increasing
669 concentrations of control IgG or M200 (50, 150 and 250 μ g/ml) on MDA-B02 cell migration through 8-
670 μ m diameter pore-size inserts coated with fibronectin. Data are the mean \pm SEM of three separate
671 experiments. **: $P < 0.001$; ***: $P < 0.0001$. **(D)** *Top-left panel*: Schematic representation of the
672 treatment protocol. MDA-B02 cells were inoculated intra-arterially to Balb/c *nude* mice. Animals
673 received a treatment with a control IgG or M200 (15 mg/kg) every other day, starting one day before
674 tumor cell inoculation. Seven days after tumor cell inoculation, animals were culled, and the bone
675 marrow collected for tumor cell colony assays. *Bottom panel*: Representative images of tumor cell
676 colonies in the bone marrow from animals treated with M200 or the control IgG.

677

678 **Figure 5:** Pharmacological inhibition of ITGA5 with M200 treatment reduces bone metastasis
679 formation *in vivo*. **(A)** *Top panel:* Schematic representation of the treatment protocol. MDA-B02 cells
680 were inoculated intra-arterially to Balb/c *nude* mice. Animals (10 mice per group) received a treatment
681 with a control IgG or M200 (15 mg/kg) every other day, starting one day before tumor cell inoculation.
682 Bone metastasis formation in animals was monitored over time by bioluminescence imaging and
683 radiography. Twenty-eight days after tumor cell inoculation, animals were culled, and metastatic bones
684 were analyzed by histomorphometry and immunohistochemistry. **(B)** *Left panels:* whole-body
685 bioluminescence imaging of a representative animal for each group at day 28. *Middle panel:*
686 Progression of tumor burden in control IgG- and M200-treated animals, as measured by whole body
687 bioluminescence imaging. *Right panel:* Kaplan-Meier estimates for rates of invasive-disease-free
688 survival (IDFS) of animals, as measured by bioluminescence imaging. **(C)** *Left panels:* radiograph of a
689 representative metastatic leg for each group at day 28. *Middle panel:* Progression of osteolytic lesion
690 areas in control IgG- and M200-treated animals, as measured by radiography. *Right panel:* Kaplan-
691 Meier estimates for rates of bone metastasis-free survival (BMFS) of animals, as measured by
692 radiography. **(D)** *Left panels:* Goldner's trichrome staining of tibial tissue sections of metastatic legs
693 from tumor-bearing mice treated with the control IgG or M200. Bone is stained green, whereas bone
694 marrow (BM) and tumor cells are stained purple. *Middle panel:* Assessment of bone destruction and
695 tumor burden as measured, respectively, by the bone volume (BV)/tissue volume (TV) ratio and tumor
696 burden (TB)/soft tissue volume (STV) ratio of metastatic legs from tumor-bearing mice treated with the
697 control IgG or M200. *: $P < 0.05$. *Right panel:* Representative Ki67 staining of tumor areas in bone
698 tissue sections from metastatic legs of animals treated with the control IgG or M200.

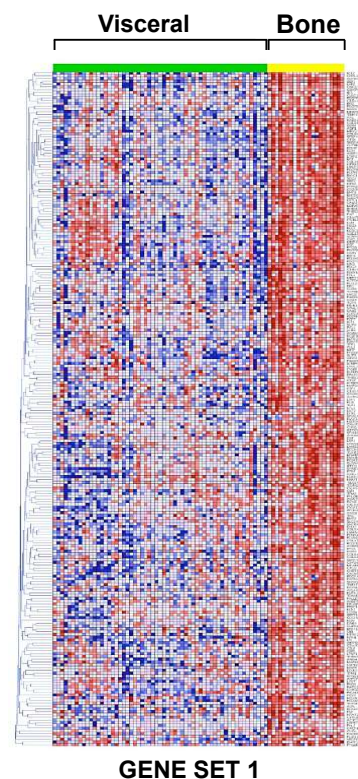
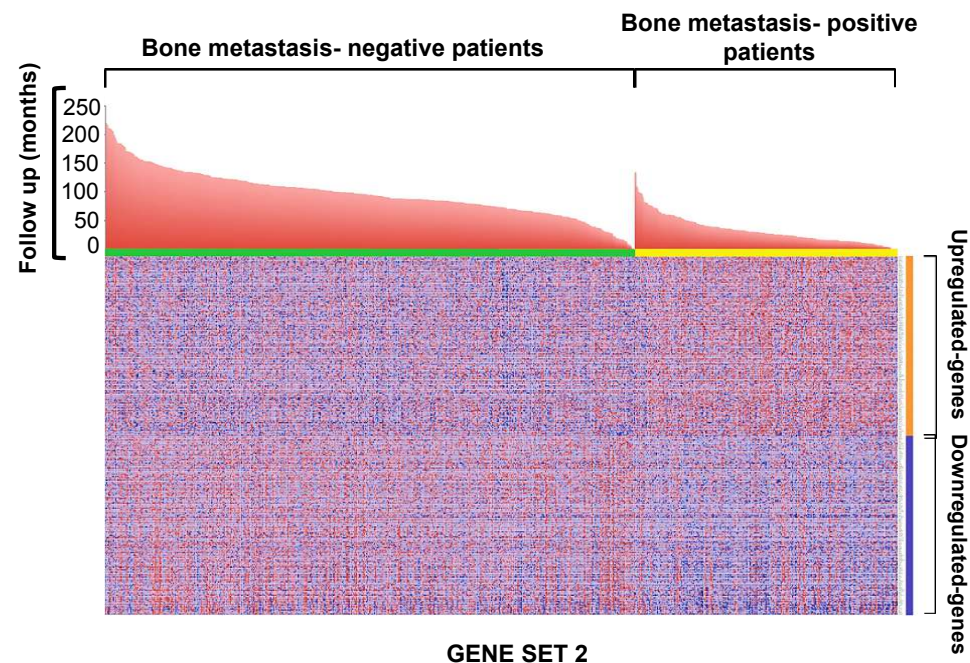
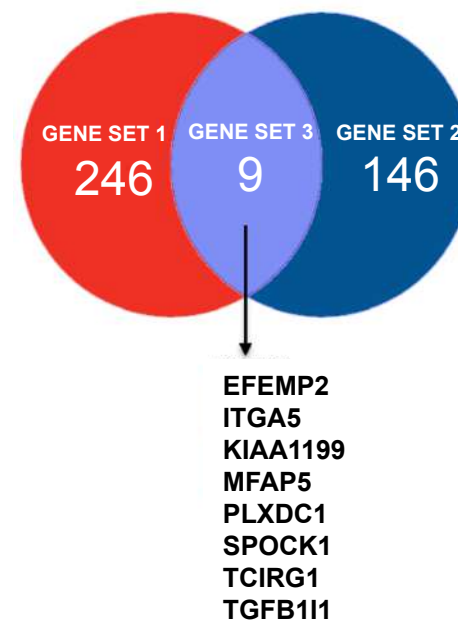
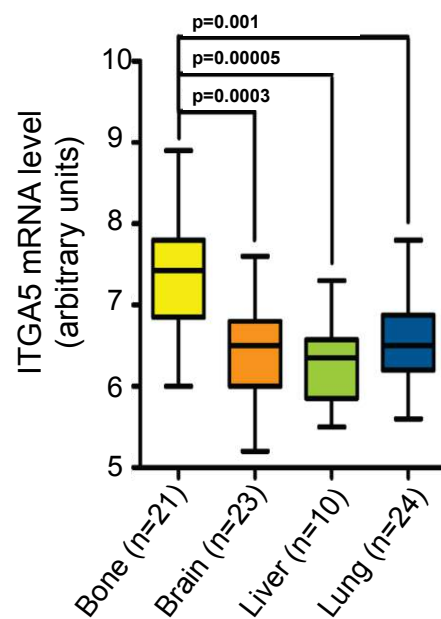
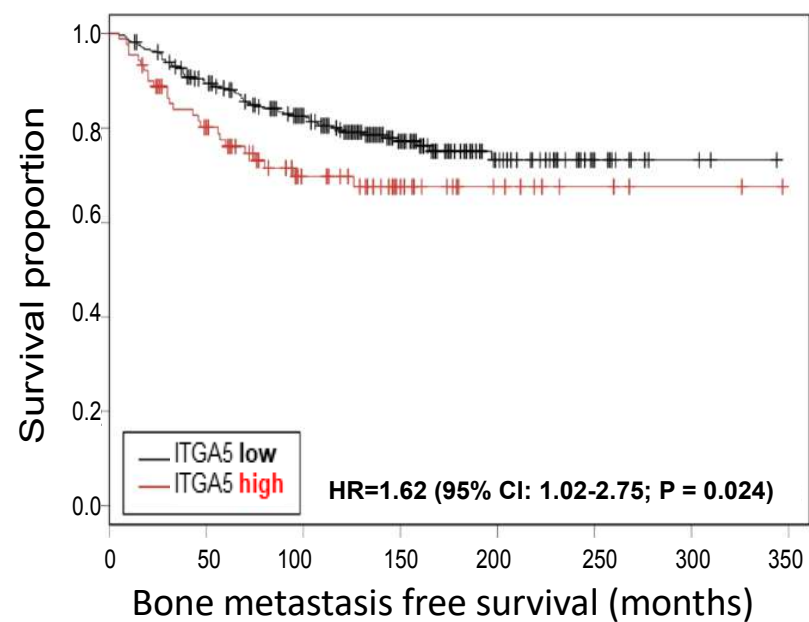
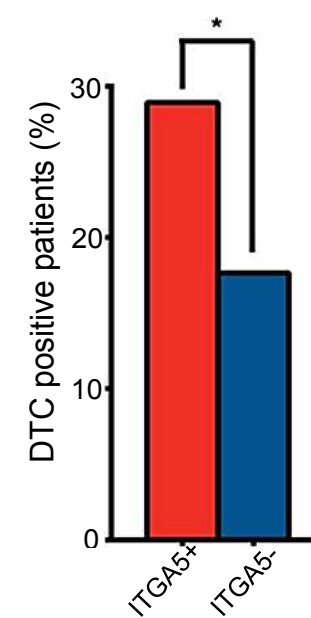
700 **Figure 6:** Pharmacological inhibition of ITGA5 with M200 treatment reduces growth of subcutaneous
701 tumor xenografts *in vivo*. **(A)** Schematic representation of the treatment protocol. MDA-B02 cells were
702 inoculated subcutaneously to Balb/c *nude* mice. Animals (5 mice per group) received a treatment with
703 a control IgG or M200 (15 mg/kg) every other day, starting one day before tumor cell inoculation.

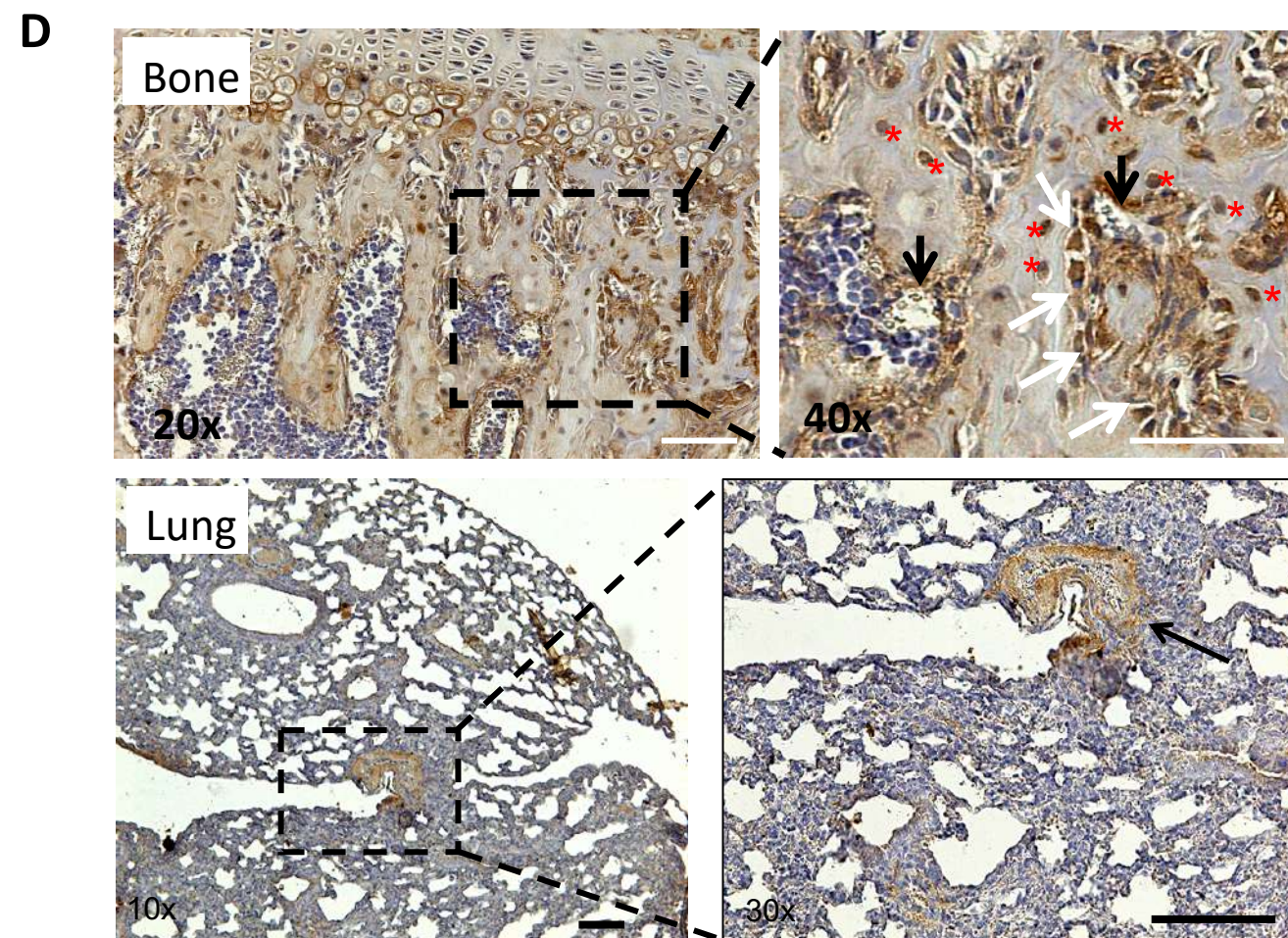
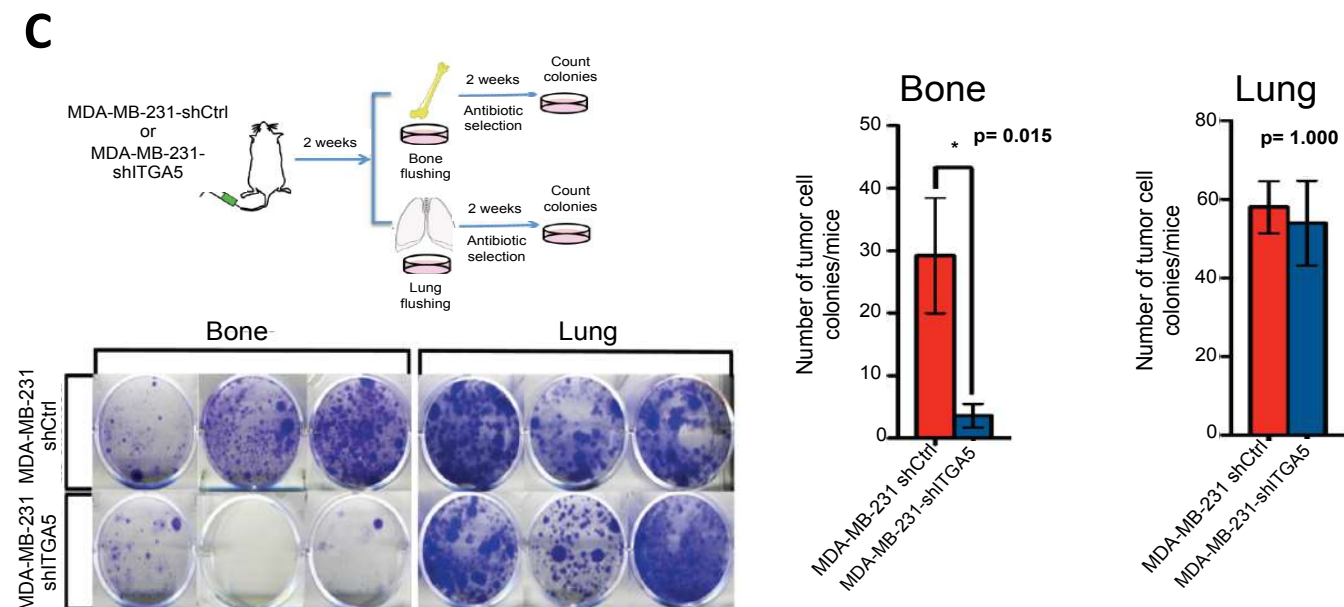
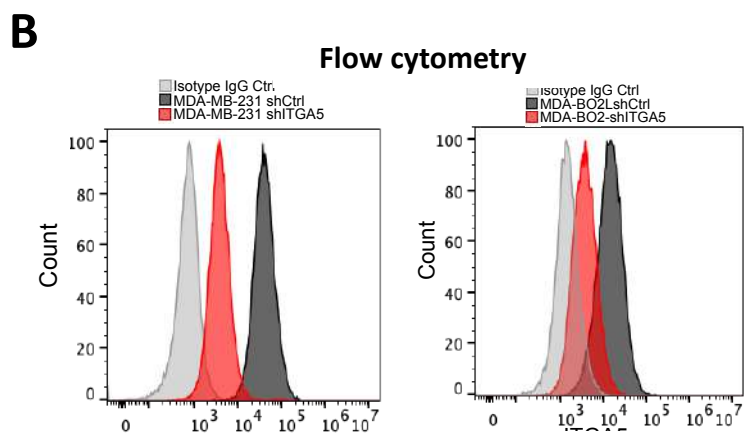
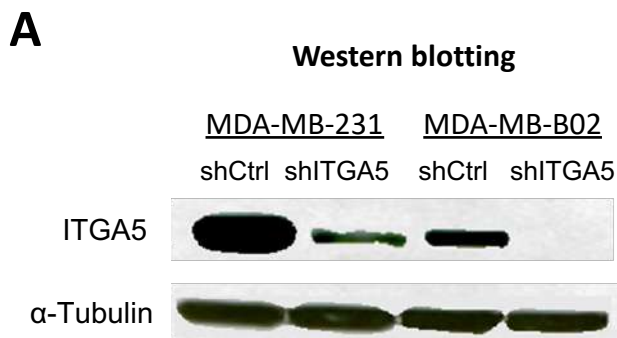
704 Tumor growth in animals was monitored over time by bioluminescence imaging and using a Vernier
705 caliper. At day 35 after tumor cell inoculation animals were culled and tumors weighted. **(B)** *Left panel:*
706 Whole-body bioluminescence imaging of 3 representative mice per group at day 35. *Right panel:*
707 Tumor burden curves of tumor-bearing animals treated with the control IgG or M200, as judged by
708 bioluminescence imaging (photons/second). **(C)** Tumor growth curves of tumor-bearing animals
709 treated with the control IgG or M200, as judged by Vernier caliper measurement (mm³). **(D)** Bar graph
710 represents the average weight of tumors for each group. Data are presented as mean ± SEM. *: *P*
711 <0.05, **: *P* <0.01, ***: *P* <0.005.

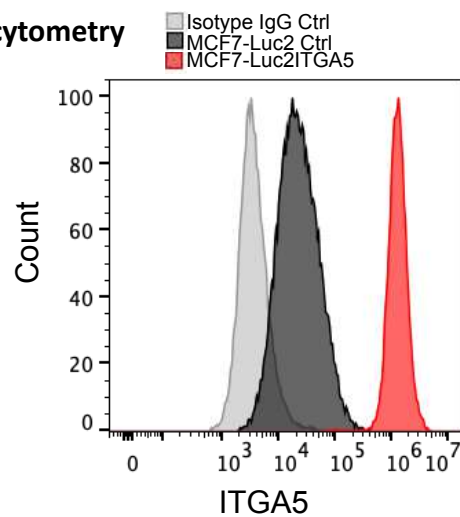
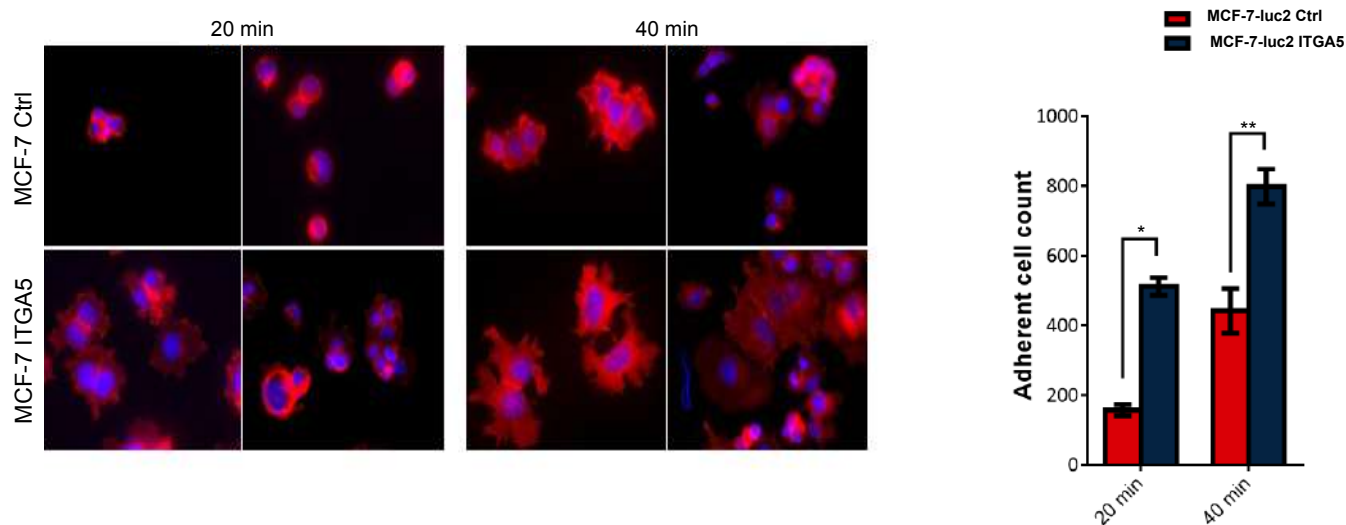
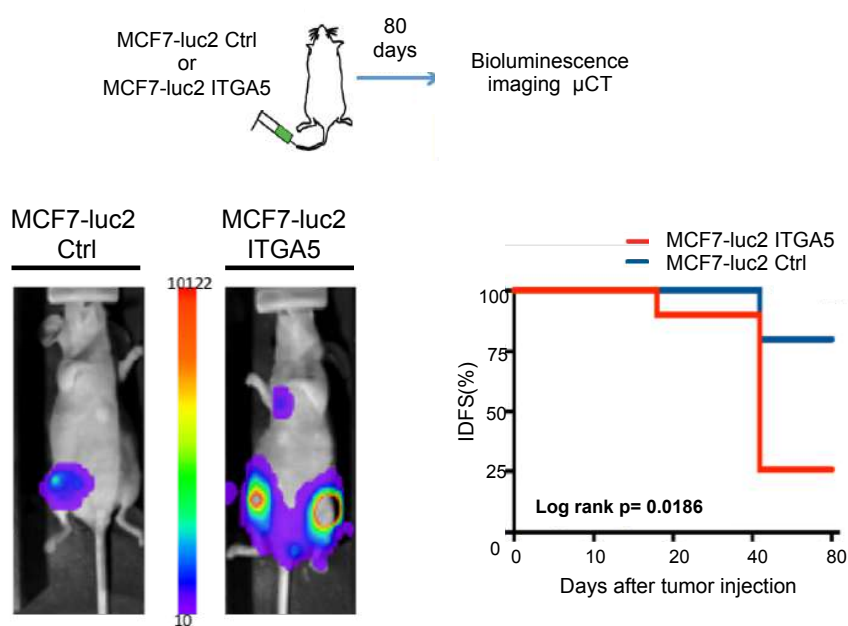
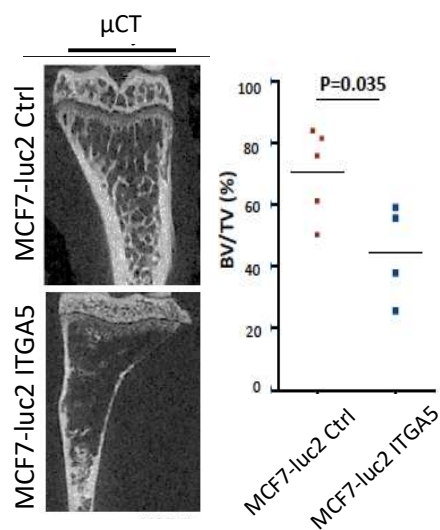
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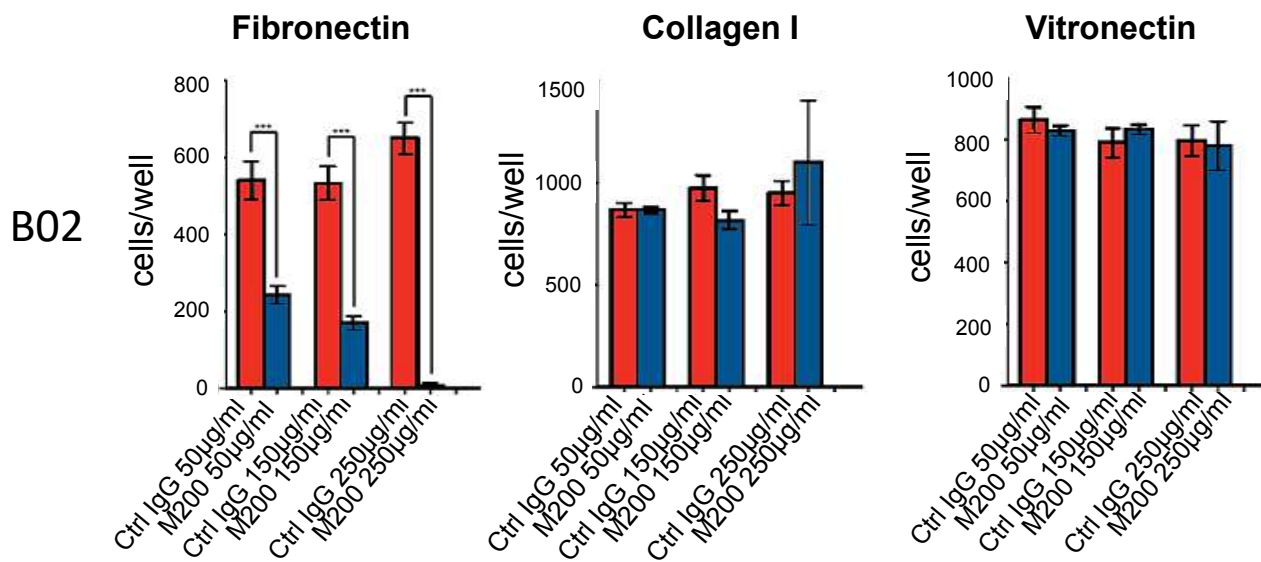
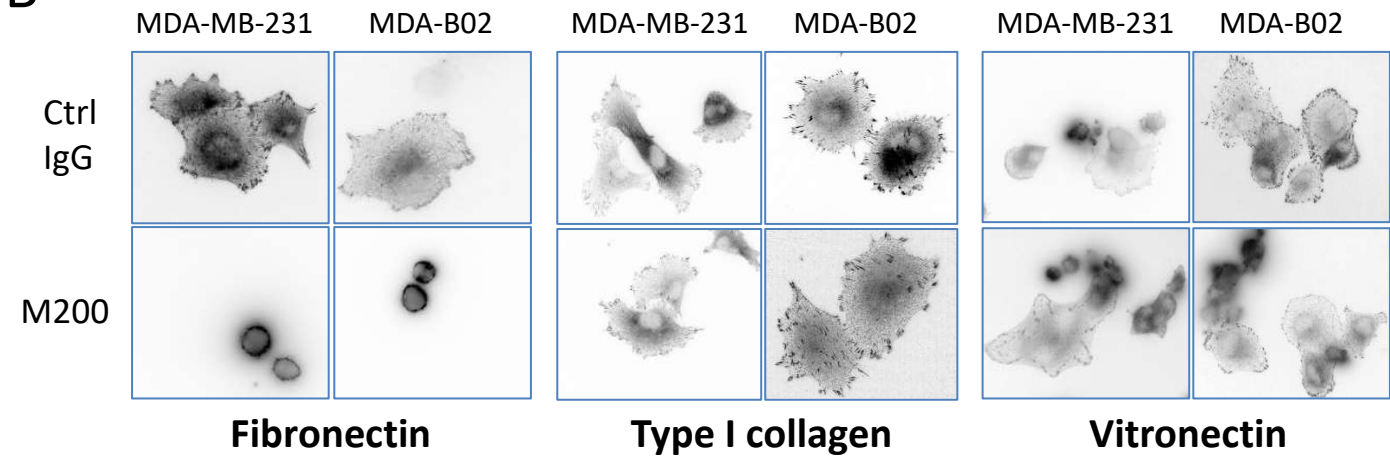
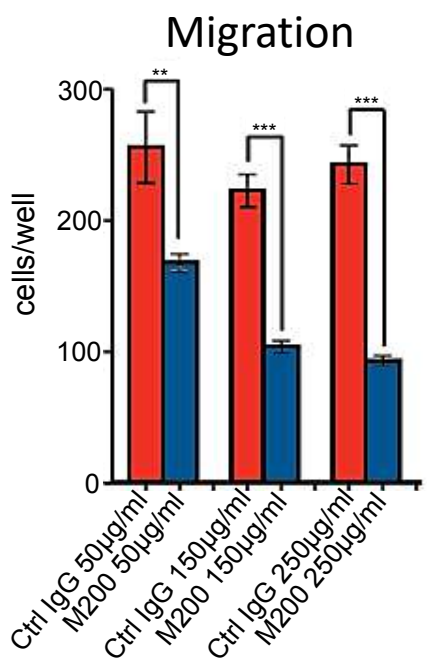
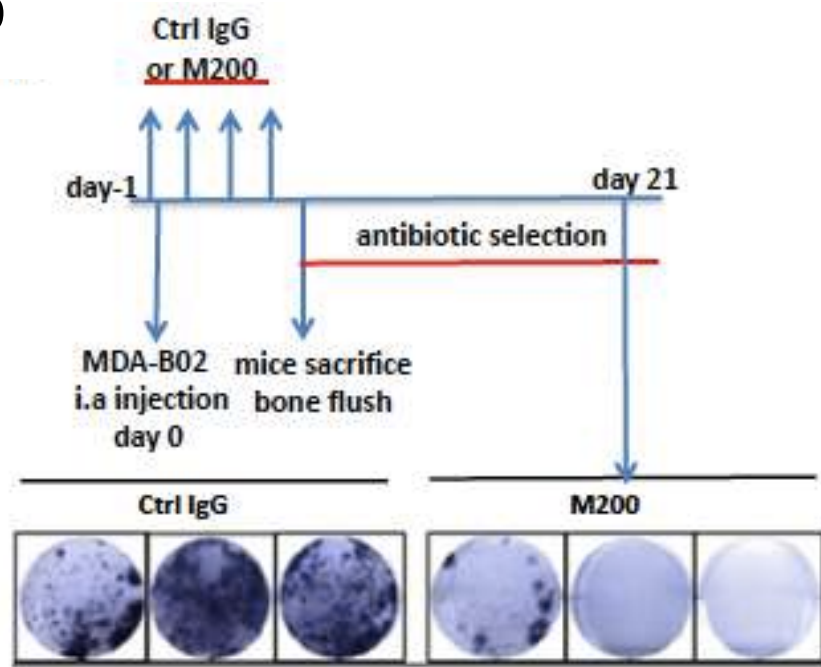
713 **Figure 7:** Pharmacological inhibition of ITGA5 with M200 antibody inhibits human osteoclast
714 differentiation and activity *in vitro*. **(A)** *ITGA5* and *cathepsin K* mRNA expression levels (relative to
715 GSUB housekeeping gene) at different stages of human osteoclast differentiation. **(B)** Western blot
716 analysis of ITGA5 at different stages of human osteoclast differentiation. **(C)** Fluorescence-based
717 staining of ITGA5 and F-actin ring (white arrowheads) at different stages of human osteoclast
718 differentiation, using phycoerythrin-conjugated anti-ITGA5 antibody and FITC-labeled phalloidin,
719 respectively. Scale bar: 100 μm. **(D)** *In vitro* osteoclast differentiation of human peripheral blood
720 mononuclear cells treated with M-CSF and RANKL, alone (Ctrl) or in combination with anti-RANKL
721 antibody denosumab (250 μg/ml) or anti-ITGA5 antibody M200 (250 μg/ml). Mature osteoclasts were
722 quantified as multinucleated (more than three nuclei), TRAP-positive cells. Representative images are
723 shown for each group. *,** : *P* < 0.05 and 0.001, respectively. **(E)** Resorption of an inorganic 3-
724 dimensional crystalline material by human osteoclasts treated with a control IgG or M200 (250 μg/ml).
725 Representative images are shown for each group. *: *P* < 0.05. **(F)** Effect of control IgG and M200 on
726 viability of human osteoclasts, as measured by MTT assay.

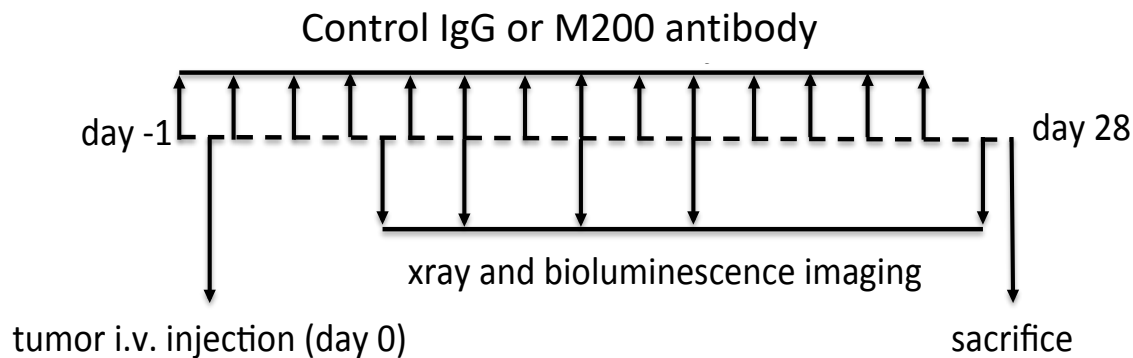
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A**B****C****D****E****F**



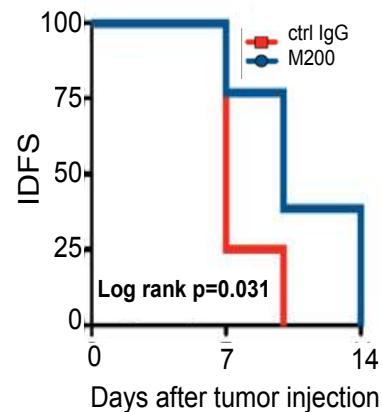
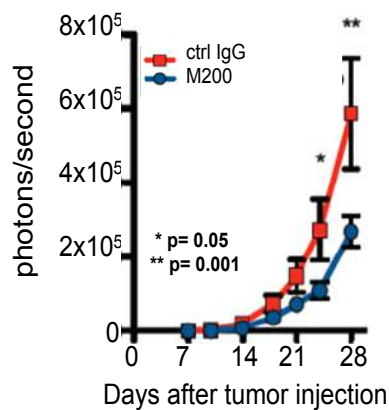
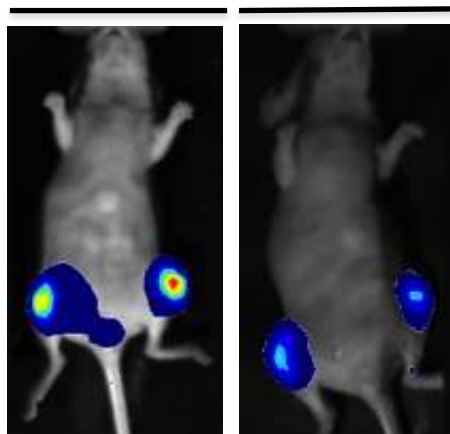
A**Western blotting****B****Flow cytometry****C****D****E**

A**B****C****D**

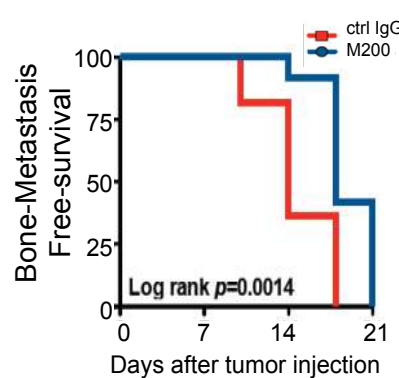
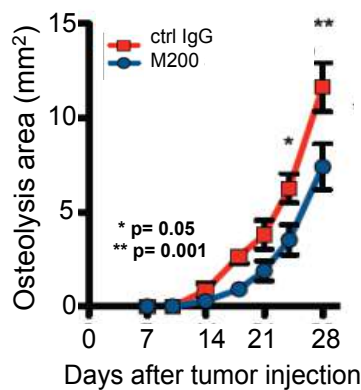
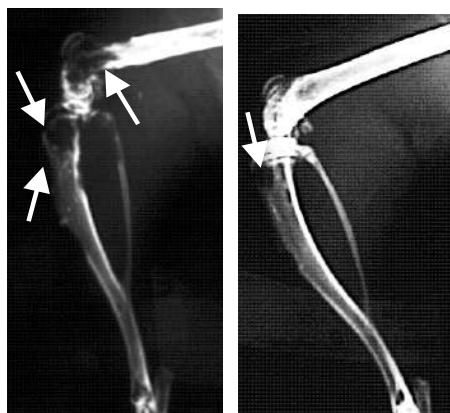
A**B**

vehicle M200

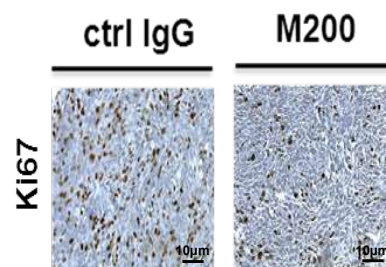
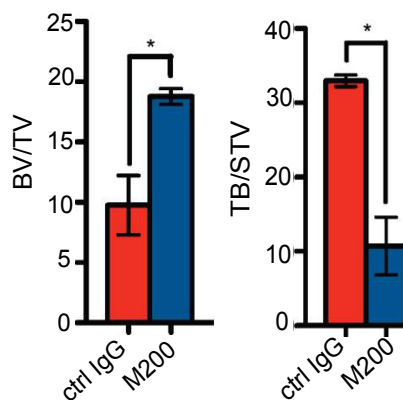
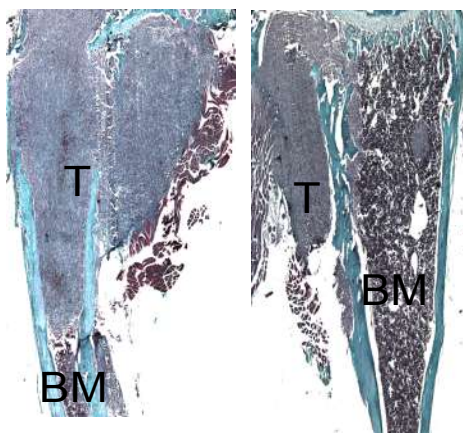
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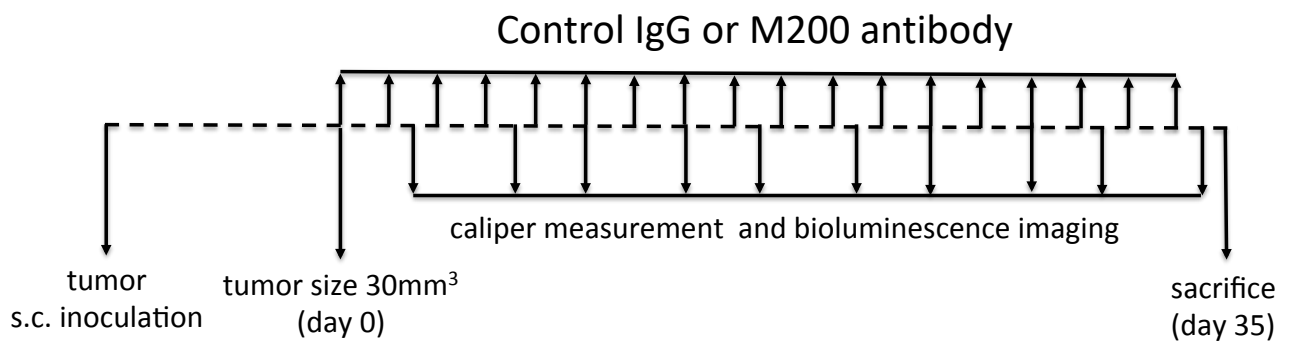
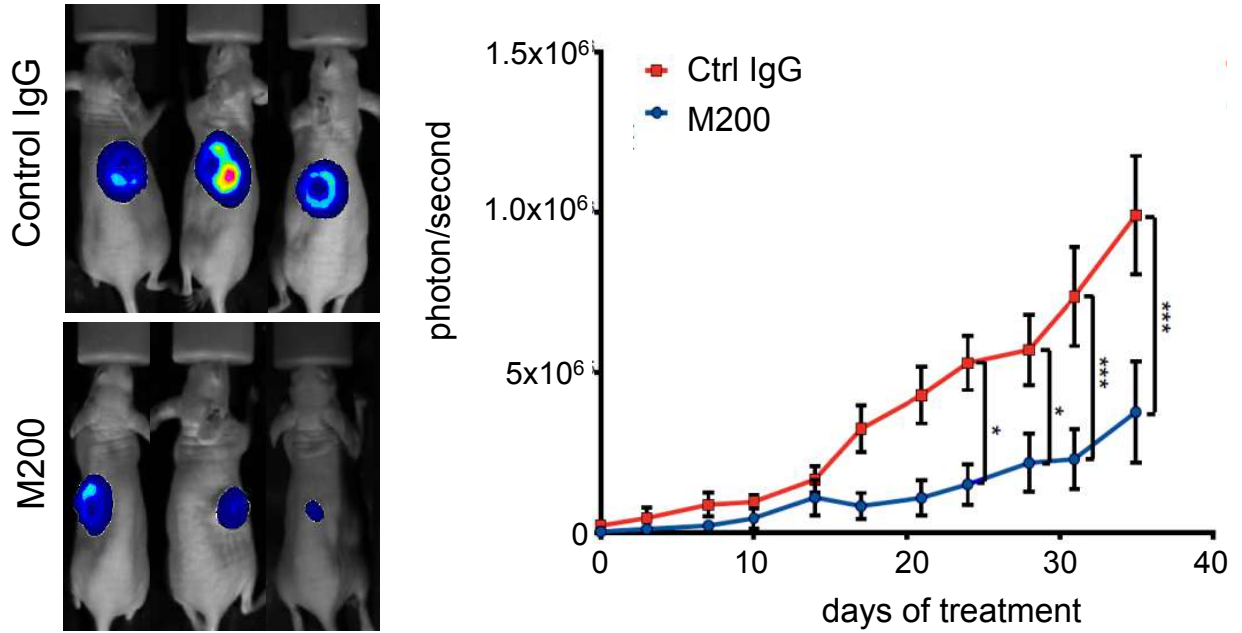
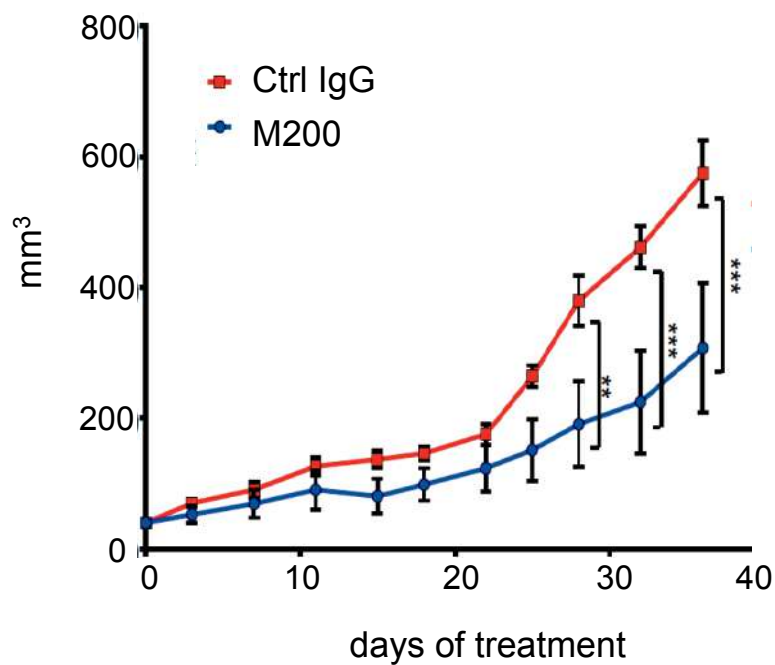
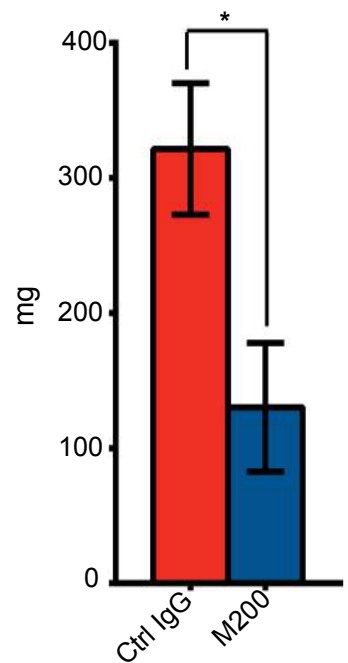
**C**

radiography

**D**

histomorphometry



A**B****C****D**

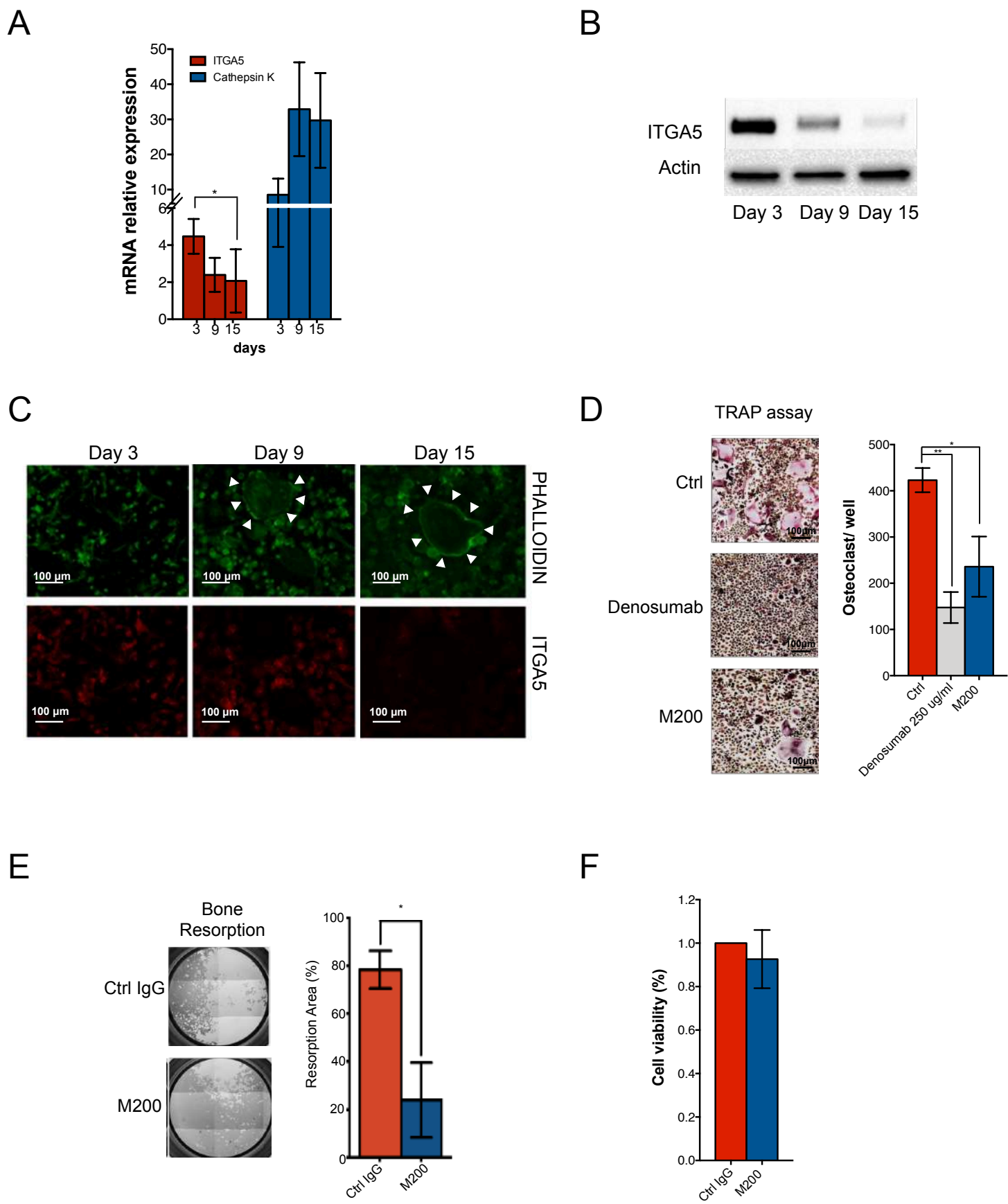


Figure 7: Pantano et al.

Table 1. Association of ITGA5 expression with clinical and biological characteristics of patients with early-stage breast cancer from the Curie Institute/Centre René Huguenin cohort.*

Characteristics	Hazard Ratio	95% CI lower limit	95% CI upper limit	P value
Age	1.000	0.985	1.016	0.965
Tumor size	1.003	0.991	1.015	0.650
Nodal status	2.635	1.271	5.462	0.009
Estrogen receptor status	0.825	0.524	1.297	0.404
Progesterone receptor status	1.267	0.858	1.872	0.234
Her2 status	0.784	0.526	1.170	0.234
ITGA5 expression	1.359	1.083	1.707	0.028

(*) Hazard ratios and 95% Confidence Intervals (CIs) are based on Cox multivariate regression analysis.