Received: 26 October 2018 | Accepted: 18 March 2019

DOI: 10.1111/jth.14443

ORIGINAL ARTICLE

jth

The direct oral anticoagulants rivaroxaban and dabigatran do not inhibit orthotopic growth and metastasis of human breast cancer in mice

Jeroen T. Buijs[1] | El H. Laghmani[1] | Rob F. P. van den Akker[1] | Chris Tieken[1] | Esther M. Vletter[1] | Kim M. van der Molen[1] | Juliette J. Crooijmans[1] | Chantal Kroone[1] | Sylvia E. Le Dévédec[2] | Gabri van der Pluijm[3] | Henri H. Versteeg[1]

[1]Einthoven Laboratory for Vascular and Regenerative Medicine, Division of Thrombosis and Hemostasis, Department of Internal Medicine, Leiden University Medical Center, Leiden, The Netherlands

[2]Division of Drug Discovery and Safety, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands

[3]Department of Urology, Leiden University Medical Center, Leiden, The Netherlands

Correspondence
Jeroen T. Buijs, Einthoven Laboratory for Vascular and Regenerative Medicine, Division of Thrombosis and Hemostasis, Dept. of Internal Medicine, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Email: j.t.buijs@lumc.nl

Funding information
Dutch Cancer Society, Grant/Award Number: #2015-7594; World Wide Cancer Research, Grant/Award Number: #15-1186

Abstract

Background: Factor Xa-targeting DOACs were recently found to reduce recurrent VTE efficiently in cancer patients when compared to the standard treatment with low-molecular-weight heparins (LMWHs). While the anticancer effects of LMWHs have been extensively studied in preclinical cancer models, the effects of FXa-targeting DOACs on cancer progression remain to be studied.

Objective: We investigated whether the FXa-targeting DOAC rivaroxaban and the thrombin-targeting DOAC dabigatran etexilate (DE) affected human breast cancer growth and metastasis in orthotopic xenograft models.

Methods/results: Mice that were put on a custom-made chow diet supplemented with rivaroxaban (0.4 or 1.0 mg/g diet) or dabigatran etexilate (DE) (10 mg/g diet) showed prolonged ex vivo coagulation times (prothrombin time [PT] and activated partial thromboplastin time [aPTT] assay, respectively). However, rivaroxaban and DE did not inhibit MDA-MB-231 tumor growth and metastasis formation in lungs or livers of 7-week-old fully immunodeficient NOD/SCID/$\gamma_C^{-/-}$ (NSG) mice. Comparable data were obtained for rivaroxaban-treated mice when using NOD-SCID mice. Rivaroxaban and DE treatment also did not significantly inhibit tumor growth and metastasis formation when using another human triple negative breast cancer (TNBC) cell line (HCC1806) in NOD-SCID mice. The FXa and thrombin-induced gene expression of the downstream target CXCL8 in both cell lines, but FXa and thrombin, did not significantly stimulate migration, proliferation, or stemness in vitro.

Conclusion: Although effectively inhibiting coagulation, the DOACs rivaroxaban and DE did not inhibit orthotopic growth and metastasis of human TNBC. It remains to be investigated whether DOACs exert antitumorigenic effects in other types of cancer.

KEYWORDS
anticoagulants, breast neoplasms, dabigatran, factor X, rivaroxaban

Manuscript handled by: Wolfram Ruf

Final decision: Pieter Reitsma, 18 March 2019

1 | INTRODUCTION

Cancer patients have a seven-fold increased risk for venous thromboembolism (VTE),[1] and this risk is dependent on treatment, patient-related factors, tumor type, and disease stage.[2,3] In patients receiving chemotherapy, it was demonstrated that thromboembolism (venous and arterial thromboembolism) is the second leading cause of death.[4] Furthermore, patients diagnosed with both cancer and VTE have a very poor prognosis when compared to patients who are diagnosed with cancer alone.[3,5]

Over the years, low-molecular-weight heparins (LMWH) and vitamin K antagonists (VKAs) have shown to be effective anticoagulants for the prevention of recurrent VTE. In cancer patients, LMWHs have been demonstrated to be more effective in inhibiting recurrent VTE than VKAs.[6] Multiple randomized clinical trials (RCTs) have been designed to study the effects of LMWHs in cancer patients with survival as primary endpoint. While some RCTs (MALT and FAMOUS[7,8]) demonstrated that LMWH treatment resulted in a survival benefit, particularly in patients with a good prognosis at entry of the study, other RCTs did not show survival benefits.[9] Altogether, a meta-analysis including mainly studies with advanced cancer patients showed a lack of survival benefit.[10]

The coagulation cascade consists of the intrinsic (contact activation) and extrinsic (tissue factor-dependent) pathways, which converge at the level of factor X (FX) activation into FXa, which in turn, in complex with active factor V (FVa), mediates the conversion of prothrombin into thrombin. In addition to activating the coagulation cascade that ultimately results in formation of a fibrin clot, FXa and thrombin can also activate protease-activated receptors on the cell membrane of platelets, endothelial and perivascular cells, directly regulating adhesion, migration and survival.[11] Increasing evidence suggests that factors of the coagulation cascade can also directly elicit protumorigenic effects by directly stimulating PARs on the cell membrane of tumor cells.[12–15] On the other hand, coagulation can also indirectly affect metastasis formation by forming fibrin/platelet-rich clots around circulating tumor cells, thereby shielding tumor cells from shear stress or natural killer (NK)-cell-mediated attack.[16,17]

LMWHs have been shown to inhibit formation of lung and bone metastases in experimental metastasis models, in which melanoma, colorectal, or breast cancer cells are directly inoculated into the circulation, either intravenously or into the left heart ventricle.[18–23] On the other hand, LMWHs did not inhibit the outgrowth of lung cancer cells when subcutaneously injected;[24] nor did they inhibit metastasis formation in an experimentally induced liver metastasis model.[25] Apart from their anticoagulant activity, LMWHs possess a variety of biological activities that may explain the observed anticancer effects, including inhibition of heparanase, blockade of P-selectin-dependent and L-selectin-dependent cell adhesion, impairment of growth factor signaling (e.g., the SDF1-CXCR4 axis), and inhibition of angiogenesis.[26–29]

The VKAs have also been shown to inhibit cancer growth at primary and metastatic sites in a preclinical pancreatic cancer model.[30] The molecular mechanism underlying the antitumor activity was, at least in part, unrelated to its effect on coagulation, but due to inhibition of Gas6-mediated activation of the Axl receptor tyrosine kinase on tumor cells.[30]

Essentials

- Factor Xa (FXa)-targeting direct oral anticoagulants (DOACs) reduce venous thromboembolism (VTE)
- The effects of FXa-targeting DOACs on cancer progression remain to be studied
- In xenograft models, a FXa-targeting DOAC did not inhibit breast cancer growth and metastasis
- A thrombin-targeting DOAC, dabigatran, also did not inhibit breast cancer growth and metastasis

As treatment with LMWH encompasses a burdensome daily i.p. injection, orally available anticoagulants have been developed; they can be classified in FXa-targeting DOACs (apixaban, edoxaban, rivaroxaban) and thrombin-targeting DOACs (dabigatran).[31,32] Both types of DOAC have shown a superior safety profile when compared to VKA and LMWH, and are at least as efficient in inhibiting VTE recurrence in patients without cancer.[33–35]

In cancer patients, it was recently shown in two RCTs that treatment with the FXa-targeting DOACs edoxaban (Hokusai VTE Cancer)[36]) and rivaroxaban (SELECT-D)[37]) resulted in a lower rate of VTE, but a higher rate of major bleedings when compared to the LMWH dalteparin,[36,37] and consequently was shown not to be non-inferior with respect to the composite outcome of recurrent VTE or major bleeding.[36] Ongoing clinical trials (e.g., CANVAS, CARAVAGGIO) are further addressing the role of FXa-targeting and thrombin-targeting DOACs in inhibiting recurrent VTE in cancer patients,[38] and these are expected to change the guidelines for treatment of cancer patients. The potential anticancer effects of these DOACs, however, are currently understudied, and potential antimetastatic effects of FXa-targeting DOACs have not been studied in preclinical cancer models. Here, we investigated whether the FXa-targeting DOAC rivaroxaban or the thrombin-targeting DOAC dabigatran affects human breast cancer growth and metastasis in xenograft models.

2 | METHODS

2.1 | Mice

All animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (LUMC). The NOD.CB17-*Prkdc*^{scid}/J, commonly known as NOD-SCID mice, and NOD.Cg-*Prkdc*^{scid}*IL2rg*^{tm1Wjl}/SzJ, commonly known as NOD-SCID gamma (NSG) mice, were purchased from Charles River. Mice were housed at the central animal facility of the LUMC at biosafety level D1, which is a multibarrier and specific-pathogen-free facility. The FELASA recommendations for the health monitoring in experimental units[39] were used. Mice were housed in Sealsafe® PLUS, green line system (Tecniplast S.p.A.) autoclaved GM500 cages, with autoclaved LIGNOCEL® BK 8-15 (BK-8-15-00433, JRS) bedding material. The

air ventilation unit inspiratory vital capacity system was + pressured with 75 air changes/h. Daytime was set at 7:00 to 18:00 with sunrise and sunset set at 6:30 to 7:00 and 18:00 to 18:30, respectively. Light intensity was 40 to 80 LUX at top of the racks and 10 to 30 LUX inside the bottom and top cages. Temperature was set at 20°C to 22°C, and water was provided ad libitum in autoclaved plastic drinking bottles (Tecniplast S.p.A.) and refreshed weekly. Based on body weight, comparable groups were made and randomly allocated to the experimental treatments using a computer-generated random number. After measurements (see 2.2), cages were placed back in random order in the rack in the animal room. Mice were fed regular chow-diet (RM3 diet, pelleted, irradiated 9 kGy, SDS diets) or custom-made chow diet (see the following) ad libitum. Custom-made chow diet was prepared by boiling 600 mL tap water with 24 g agar (Sigma-Aldrich), adding 600 mL sterile aqua dest, and subsequently mixing well with 1200 g diet powder (RMH-B, 9 kGy irradiated, ABDiets, Woerden, The Netherlands). Chow diet was freeze-dried for at least 3 days, and stored at −20°C until further use. For the specific experimental groups, rivaroxaban (Bayer) and DE (Boehringer Ingelheim) were purchased at the pharmacy at the LUMC, and the pills were ground, weighed, and carefully mixed in the chow diet at concentrations of 0.4, 1, or 10 mg/g chow diet, before freeze drying. As mice are nocturnal animals, blood was collected in the morning when peak plasma levels of diet-fed anticoagulants are expected.

2.2 | Orthotopic breast cancer models

The MDA-MB-231 and MDA-MB-231-LC cells (see 2.4) (1×10^6 in 50 μL PBS) were injected into the fourth mammary fat pad of female NOD-SCID or NSG mice (as described in detail in reference [40]). HCC1806 cells (2×10^6 in matrigel:PBS, 1:1) were injected into the fourth mammary fat pad of 7-week-old female NOD-SCID mice. Tumor volume was monitored by weekly or biweekly caliper measurements using the formula for tumor volume: (length × width × width)/2. In all experiments, six animals per group were used. At the end of the experiments, mice were sacrificed and orthotopic tumors were excised and weighed. Tumors, lungs (left), and livers were snap frozen in liquid nitrogen, and stored at −80°C until further processing for RNA.

2.3 | Cell culture

The human TNBC cell lines MDA-MB-231 and HCC1806 were derived from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 cells were cultured in DMEM 1× (Dulbecco's Modified Eagle Medium, 4.5 g/L D-glucose, L-glutamin, pyruvate; ThermoFisher Scientific, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (pen-strep). HCC1806 cells were cultured in RPMI1640 (L-arginine, L-glutamine, L-lysine; ThermoFisher Scientific) supplemented with 10% FBS and 1% pen-strep. Cells were trypsinized with triplE Express (ThermoFisher Scientific). Cells were grown in an incubator at 37°C with 5% CO_2.

2.4 | Generation of the MDA-MB-231 lung-metastatic clone

To generate GFP-positive cells, MDA-MB-231 cells were stably transduced with the MISSION® pLKO.1-puro-CMV-TurboGFP™ plasmid (Sigma-Aldrich) using the 2nd generation lentiviral packaging system, using psPAX2 and pVSVG vectors. Forty-eight hours after transduction, cells were put on puromycin selection (1 μg/mL). The MDA-MB-231-GFP cells were orthotopically injected as described in NOD-SCID gamma mice, and 12 weeks later lung metastases were detected, excised, grown in vitro under puromycin selection, and microscopically validated for a positive GFP signal. This newly generated subclone of MDA-MB-231 was named "MDA-MB-231-lung clone" (MDA-MB-231-LC) and demonstrated in a subsequent round of orthotopic injection increased lung metastatic capability when compared to its parental counterpart (see Figure S1).

2.5 | Coagulation assays

The PT was determined by adding 33.7 μL diluent (0.1% BSA in 20 mmol/L HEPES, 150 mmol/L NaCl, 0.1% PEG-8000, 5 mmol/L CaCl2, pH 7.5) to a 16.3 μL plasma sample, followed by a 60-s incubation period at 37°C. Coagulation was initiated after addition of 50 μL Neoplastine (Neoplastine CI Plus 10 reagent, Diagnostica Stago, Paris, France) of 25 mmol/L $CaCl_2$, and the coagulation time was monitored using a Start4 coagulation instrument (Diagnostica Stago).

The aPTT was determined by adding 12.5 μL Owren–Koller diluent to a 12.5 μL plasma sample and mixed with 25 μL aPTT reagent (TriniCLOT automated aPTT, Kordia), followed by a 180-sincubation period at 37°C. Coagulation was initiated after the addition of 25 μL of 25 mmol/L $CaCl_2$, and measured with the Start4 coagulation instrument.

In a chromogenic FXa assay, plasma samples and seven different spiked rivaroxaban concentrations in NPP (500, 250, 125, 62.5, 31.3, 15.6, and 8.3 ng/ml) were 11× serially diluted (in 20 mmol/L Hepes, 150 mmol/L NaCl, 0.1% PEG8000, pH 7.5). Recombinant human FXa and Spectrozyme Fxa American Diagnostica Inc (Stamford, CT; now Sekisui Diagnostics) were added to reach a final concentration of 5 nmol/L and 250 μmol/L, respectively. Factor Xa activity was subsequently measured by using a kinetic recording spectrophotometer.

2.6 | Ribonucleic acid, complementary deoxyribonucleic acid, and quantitative polymerase chain reaction

To determine gene expression (CXCL8, VEGFA), 300 000 cells were seeded per 6-well in regular culture medium. The following day, the medium was aspirated and replaced by serum-free medium. After 24 h of serum starvation, cells were stimulated with FXa (Haematologic Technologies Inc., Essex Junction, VT) or thrombin

(Enzyme Research Laboratories, South Bend, IN) in the presence or absence of rivaroxaban (Alsachim, Ilkirch Graffenstaden, France) or dabigatran (Alsachim). Cell lysate was collected in 1 mL Trisure (TRIsure, Bioline, Taunton, MA).

Total RNA of all in vitro and in vivo samples was isolated from Trisure and converted to cDNA using Super Script II using manufacturer's instructions (ThermoFisher Scientific). The qPCRs were performed in a 10-μL reaction, with SYBRselect master mix (Thermofisher Scientific), 20 ng cDNA template, and 200 nmol/L primers (see Table S1 for primer sequences), in a 384-well-qPCR plate on a CFX384 Touch Real-Time PCR detection system (BioRad, Veenendaal, The Netherlands). After 10 minutes preheating at 95°C, 40 cycles were run of 15 seconds 95°C and 60 seconds 60°C. The C(t)-values were obtained using a threshold of 150 relative units using BioRad CFX-software. Technical triplicates were run for every sample.

2.7 | Proliferation, clonogenic, and sphere assays

The MTS proliferation assay was performed as described by the manufacturer's protocol (Promega, Leiden, The Netherlands). In short, cells were seeded in a 96-well plate and culture medium was replaced the next day by medium containing 0% or 10% FBS. Coagulation factors or vehicle solution was added 3 h later and cell viabilty was checked after 3 and 7 days.

In the clonogenic assay, 100 cells/well were seeded in a 6-well plate in culture medium, and coagulation factors or vehicle was added the subsequent day. The number of colonies was counted manually 7 (MDA-MB-231) and 14 (HCC1806) days later using an inverted laboratory microscope (Leica DM IL LED). In the mammosphere assay, cells were resuspended three times using a 25G needle and 100 cells/96-well were seeded in a low-attachment plate in DMEM/F12 phenol-red free medium supplemented with 1× B-27 (minus vitamin A, ThermoFisher Scientific) and 20 ng/mL hEGF (ThermoFisher Scientific). Pictures were taken with a digital microscope camera (Leica DFC295) 8 days later. Mammospheres with a diameter larger than 50 μmol/L (using Image J for analysis) were counted, and mammosphere forming efficiency is shown as the number of mammospheres/cells seeded.

2.8 | Live cell imaging-based random cell migration assay

A live cell imaging-based random cell migration assay was performed as previously described.[41] In short, 10 000 MDA-MB-231 and HCC1806 cells were seeded in medium containing 10% FBS in a 96-well in 200 μL. The next day, cells were incubated with Hoechst 33342 (Sigma-Aldrich; 0.1 μg/mL) for 1 h to allow for nuclear cell tracking. Then, the medium was replaced by medium containing either 0% FBS or 10% FBS, for the respective 0% FBS and 10% FBS conditions. Cells were stimulated 3 h later.

To allow for automated nuclear cell tracking the 96-well plate was placed on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 20× objective (0.75 NA, 1.00 WD), and perfect focus system. Automatically, two positions per well were defined, and the signal was acquired at two positions every 600 seconds, for a total imaging period of 24 hours. All data were converted and analyzed using custom-made ImagePro Plus (MediaCybernetics, Rockville, MD, USA) macros.[42] Averages are shown from two independent experiments.

2.9 | Statistical analysis

Statistical testing was performed as described in detail in the respective figure legends. A P value of $P < 0.05$ was considered significant. Averages ± SEM are shown. GraphPad prism version 7 was used for statistical analysis.

3 | RESULTS

To study the effects of DOACs on breast cancer growth and metastasis, we tested the effects of two types of clinically available DOACs, the FXa-targeting rivaroxaban and the thrombin-targeting DE, in orthotopic breast cancer models. As a proof of principle, relatively high doses of rivaroxaban (0.4 mg/g chow diet) and DE (10 mg/g chow diet) were mixed in custom-made chow diet, which were previously shown to prolong ex vivo coagulation time in a model of sickle cell disease.[43] In the current study, we first demonstrated that NSG mice that were fed chow diet containing rivaroxaban or DE had significantly increased coagulation times as measured by PT and aPTT assay, respectively (Figure 1A,B). In addition, plasma from rivaroxaban-treated mice showed decreased FXa activity in an ex vivo chromogenic assay (Figure 1C). Neither rivaroxaban nor DE affected orthotopic tumor growth of MDA-MB-231 cells as shown by weekly caliper measurements and tumor weight at the end of the experiment (Figure 1D,E). To detect metastatic human breast cancer cells in lungs and livers, human-specific GAPDH primers were used in a qPCR and normalized to mouse β-actin. To examine the detection limit of this human-specific qPCR assay, 100 000, 1000, 10, and 0 MDA-MB-231 human breast cancer cells were spiked in whole mouse lungs. While the human-specific GAPDH product was detected in all lungs that were spiked with cancer cells, no human GAPDH product was detected in lungs that were not inoculated with cancer cells (Figure S2). The lower detection limit of detecting human cancer cells in mouse lungs using this qPCR assay is therefore at least 10 cancer cells. In NSG mice, relatively high levels of human cancer cells were detected in lung and liver as assessed by the human-specific qPCR (Figure 1F,G). However, rivaroxaban or DE treatment did not significantly inhibit the metastatic burden in both organs (Figure 1F,G). The mRNA expression levels of human SOX9 in the tumor were reduced in the rivaroxaban-treated mice when compared to vehicle and DE-treated mice (Figure 1H).

The NSG mice do not have functional NK cells and it cannot be excluded that the lack of an inhibitory effect of DOACs on metastasis formation was due to the absence of active NK cells. In contrast to NSG mice, NK cells can be activated in NOD-SCID mice.[44]

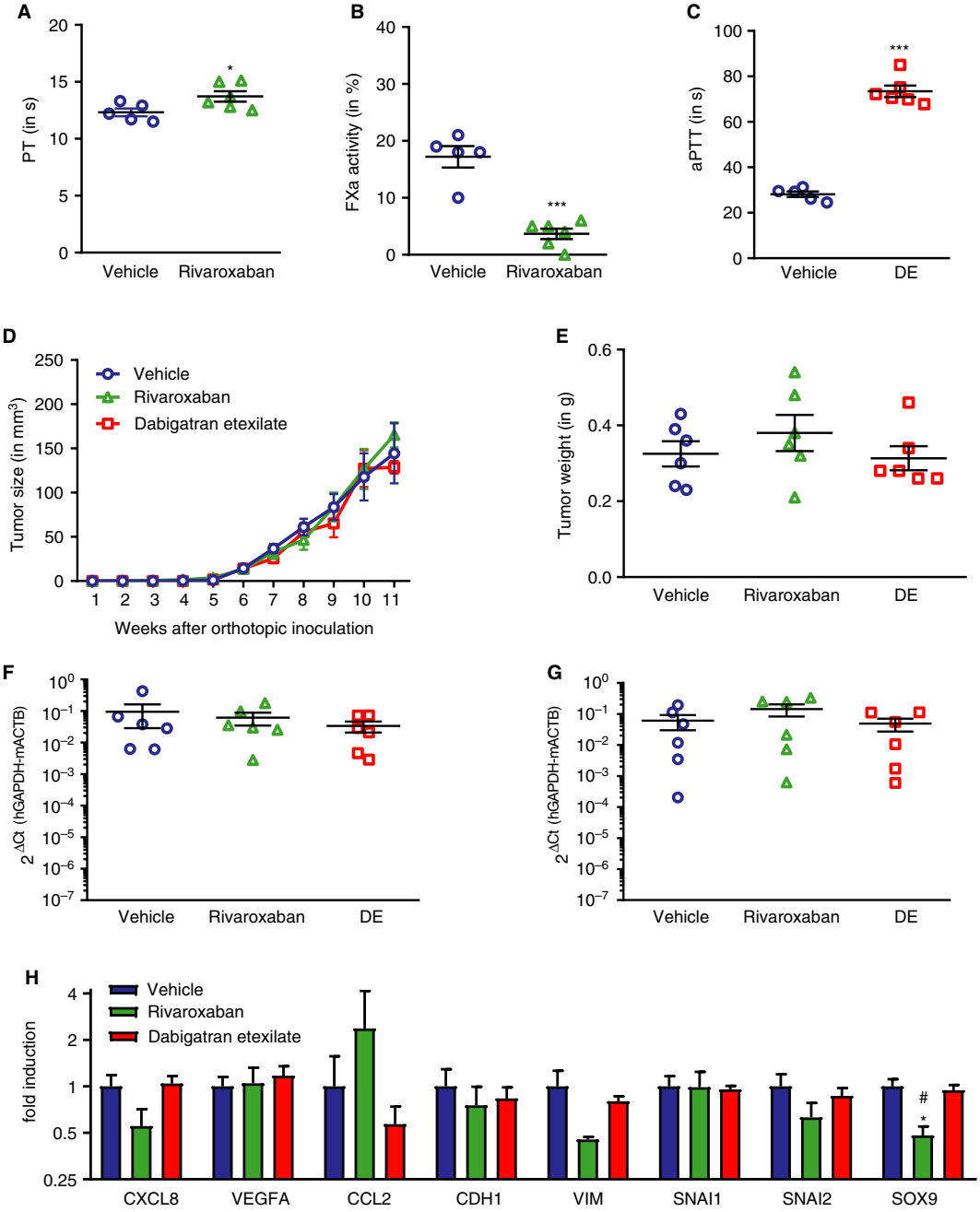

FIGURE 1 Effects of the FXa-targeting DOAC rivaroxaban and the thrombin-targeting DOAC dabigatran etexilate (DE) on orthotopically growing MDA-MB-231 breast cancer cells in NOD-SCID gamma (NSG) mice. Mice that were fed chow diet containing rivaroxaban (0.4 mg/g chow diet) and DE (10 mg/g chow diet) had significantly increased coagulation times as measured by PT (A) and aPTT (C) assay respectively. B, Plasma from rivaroxaban mice inhibited FXa activity in an ex vivo chromogenic assay. Based on spiked concentrations of rivaroxaban in normal pooled plasma, the average plasma concentration of rivaroxaban was estimated at 177 ng/mL. Neither rivaroxaban nor DE affected orthotopic tumor growth as shown by weekly caliper measurements (D) and tumor weight (E) at the end of the experiment. Sensitive human-specific qPCR showed that rivaroxaban and DE did not significantly inhibit metastasis to lung (F) and liver (G). Two livers and lungs from mice that were not inoculated with cancer cells served as a negative control that remained negative for a human-specific GAPDH product. H, Gene expression in the orthotopic tumor as measured by qPCR using human specific primer sets. Gene expression was normalized to two housekeeping genes (GAPDH and ACTB) and shown as fold induction to vehicle-treated mice. N = 6 mice per experimental group. Unpaired student *t* test (A,B,C), 1-way ANOVA with Tukey's multiple comparison test (E,F,G) 2-way ANOVA with Tukey's multiple comparison test (D) and 1-way ANOVA with Dunnett's test with additional original false discovery rate method of Benjamini and Hochberg method to correct for multiple testing of the 8 genes (H) were used for statistical evaluation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Vehicle, $^{\#}P < 0.05$ vs DE. PT, prothrombin time; aPTT, activated partial thromboplastin time; DE, dabigatran etexilate; DOAC, direct oral anticoagulant; FXa, factor Xa; qPCR, quantitative polymerase chain reaction

In addition, Dewan and coworkers demonstrated that s.c. injected MDA-MB-231 breast cancer cells metastasize to distant organs in NSG mice, but not in NOD-SCID mice.[45,46] When NOD-SCID mice were treated with the TMβ1 antibody, which transiently abrogates NK-cell activity, the s.c. injected MDA-MB-231 cells metastasized to distant organs, demonstrating that NK cells in NOD-SCID mice are functionally active in targeting breast cancer cells.

In a comparable experimental setup as described for Figure 1, we tested the effects of rivaroxaban treatment on tumor growth and metastasis in NOD-SCID mice (Figure 2). Coagulation parameters were also significantly affected by rivaroxaban in NOD-SCID mice (Figure 2A,B). When comparing control NOD-SCID mice to control NSG mice, we observed that while growth at the orthotopic site was only slightly reduced (Figures 1D,E and 2C,D), the metastatic load in

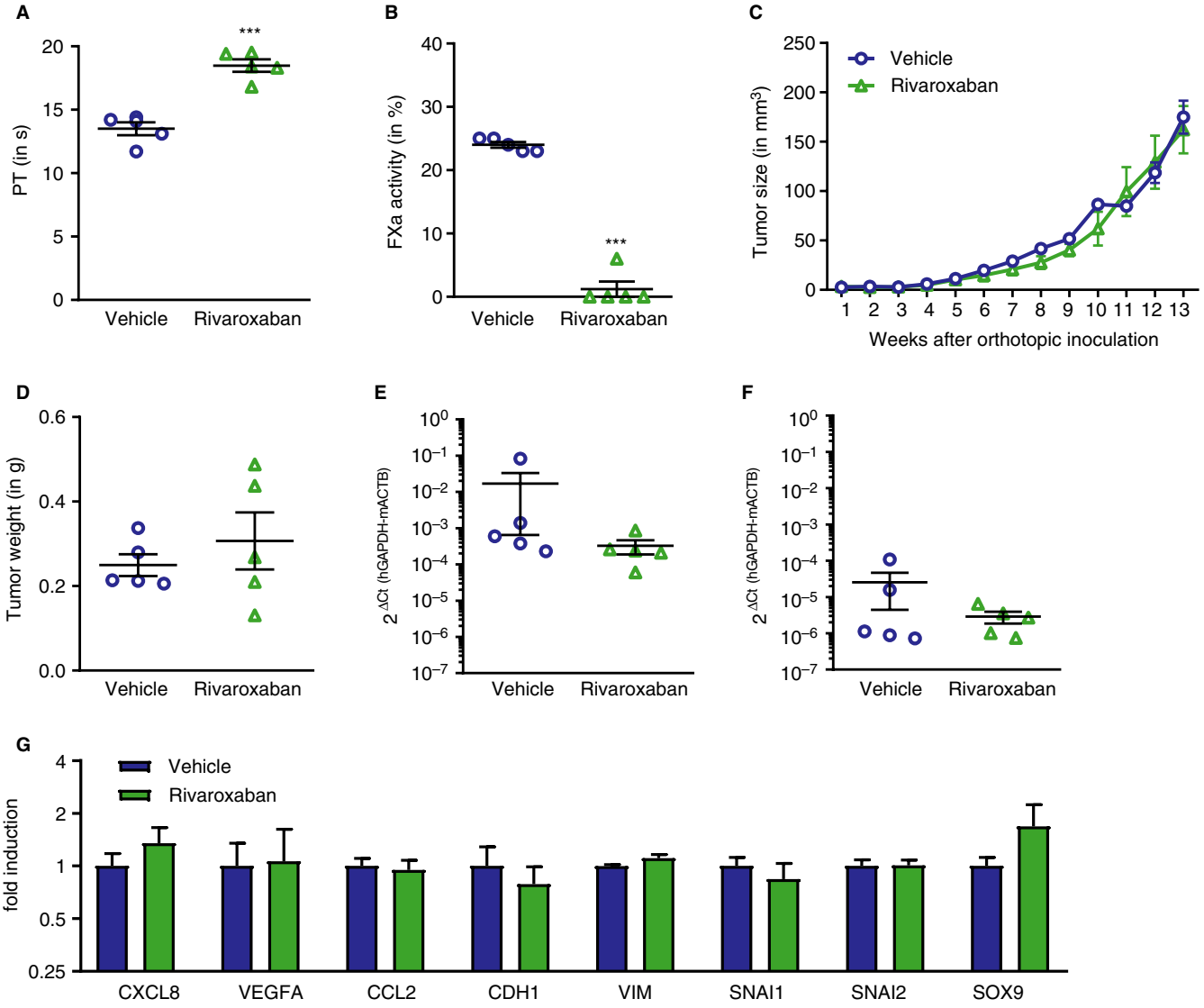

FIGURE 2 Effects of the FXa-targeting DOAC rivaroxaban on orthotopically growing MDA-MB-231 breast cancer cells in NOD-SCID mice. D, Mice that were fed chow diet containing rivaroxaban (0.4 mg/g chow diet) had significantly increased coagulation times as measured by PT assay. B, Plasma from rivaroxaban mice inhibited FXa activity in an ex vivo chromogenic assay. Based on spiked concentrations of rivaroxaban in normal pooled plasma, the average plasma concentration of rivaroxaban was estimated at 239 ng/mL. Rivaroxaban did not affect orthotopic tumor growth as shown by weekly caliper measurements (C) and tumor weight (D) at the end of the experiment. Sensitive human-specific qPCR showed that DE and rivaroxaban did not significantly inhibit metastasis in lung (E) and liver (F). Two livers and lungs from mice that were not inoculated with cancer cells served as a negative control that remained negative for a human-specific GAPDH product. (G) Gene expression in the orthotopic tumor as measured by qPCR using human specific primer sets. Gene expression was normalized to two housekeeping genes (human GAPDH and human ACTB) and shown as fold induction to vehicle mice. N = 6 per experimental group; 1 mouse in vehicle group died during the experiment; 1 mouse in rivaroxaban group did not develop an orthotopic tumor; both mice were excluded from all further analyses. A 2-tailed unpaired student's *t* test (A,B,D,E,F) with, additional original false discovery rate method of Benjamini and Hochberg to correct for multiple testing of the 8 genes (G), and a 2-way ANOVA using Sidak's multiple comparison test (C) were used for statistical evaluation. ***$P < 0.001$ vs vehicle. DE, dabigatran etexilate; DOAC, direct oral anticoagulant; FXa, factor Xa; PT, prothrombin time; qPCR, quantitative polymerase chain reaction

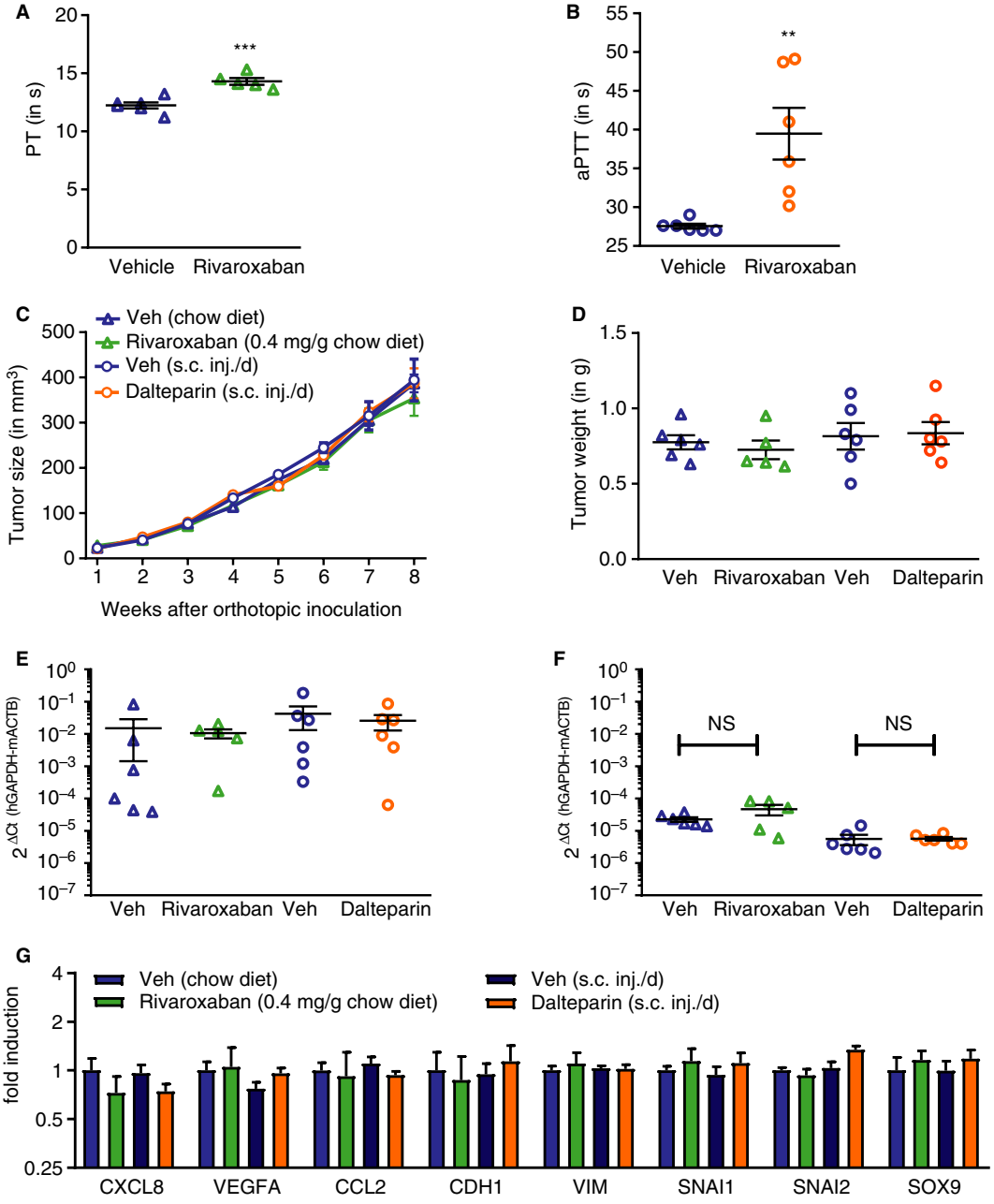

FIGURE 3 Effects of rivaroxaban and the low-molecular-weight heparin dalteparin on orthotopically growing MDA-MB-231-LC breast cancer cells in NOD-SCID mice. Mice that were fed custom-made chow diet containing rivaroxaban (0.4 mg/g chow diet) or mice that received treatment with dalteparin (200 IU/kg/d, 1× s.c. injection/day) had significantly increased coagulation times as measured by PT (A) and aPTT (B) assay, respectively. Neither rivaroxaban nor dalteparin affected orthotopic tumor growth as shown by weekly caliper measurements (C) and tumor weight (D) at the end of the experiment. Sensitive human-specific qPCR showed that rivaroxaban and dalteparin did not significantly inhibit metastasis in lung (E) and liver (F). Two livers and lungs from mice that were not inoculated with cancer cells served as a negative control that remained negative for a human-specific GAPDH product. G, Gene expression in the orthotopic tumor as measured by qPCR using human specific primer sets. Gene expression was normalized to two housekeeping genes (GAPDH and ACTB) and shown as fold induction to the "Vehicle (custom-made chow diet)" mice. In all analyses, rivaroxaban-treated and dalteparin-treated mice were compared to their own control groups: "vehicle (custom-made chow diet)" and "vehicle (1× s.c injection/day)," respectively, and statistically tested by a D-F, 2-tailed unpaired student's *t* test (A-B, D-F) with additional original false discovery rate method of Benjamini and Hochberg to correct for multiple testing of the 8 genes (G) or a 2-way ANOVA using Sidak's multiple comparison test (C). N = 6 per experimental group; 1 mouse in rivaroxaban group did not develop an orthotopic tumor and was excluded from all further analyses. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs respective Vehicle; NS, not significant. aPTT, activated partial thromboplastin time; PT, prothrombin time; qPCR, quantitative polymerase chain reaction

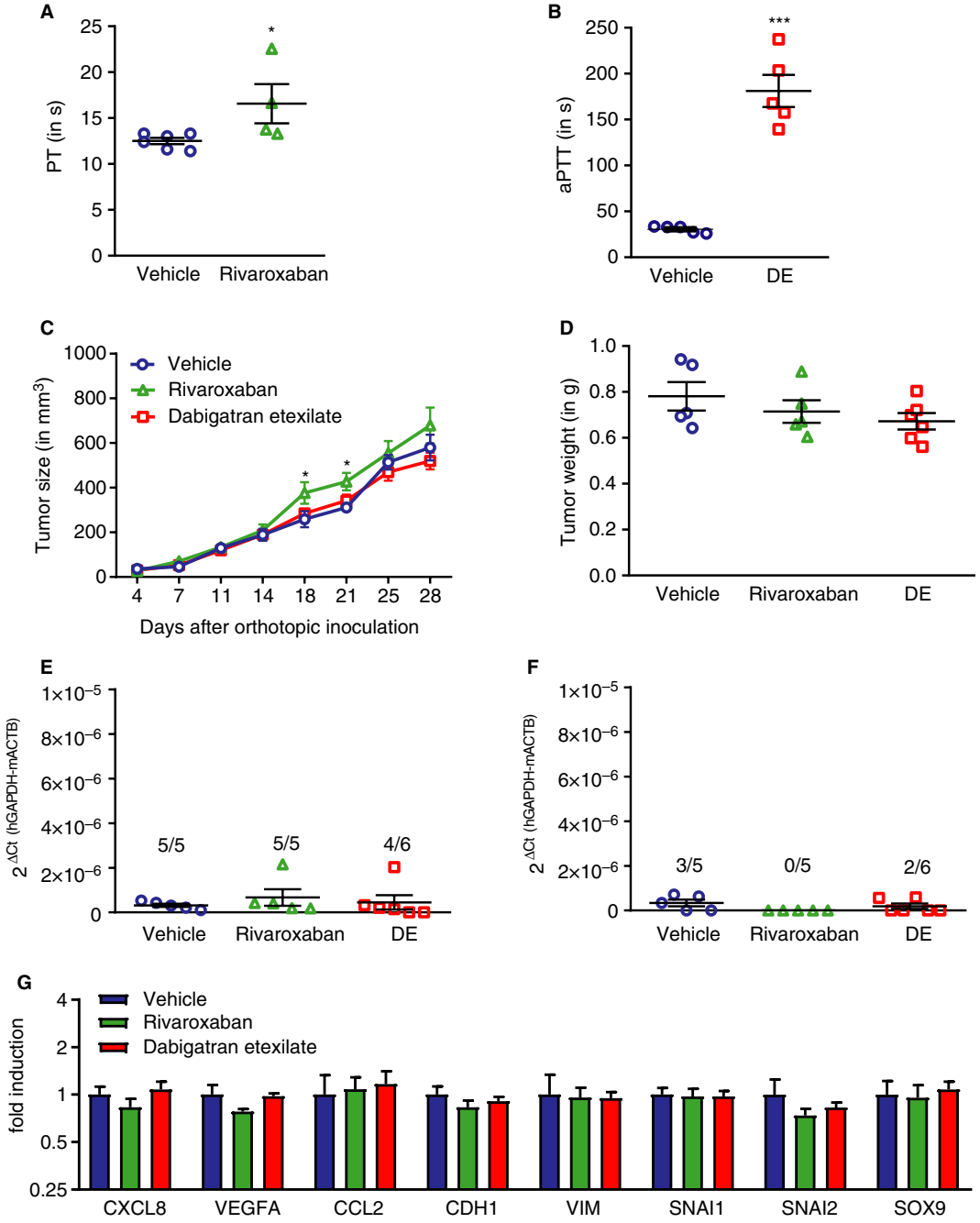

FIGURE 4 Effects of the direct oral anticoagulants rivaroxaban and dabigatran etexilate on orthotopically grown HCC1806 breast cancer cells in NOD-SCID mice. Mice that were fed custom-made chow diet containing rivaroxaban (1.0 mg/g chow diet) or dabigatran etexilate (DE) (10 mg/g chow diet) had significantly increased coagulation times as measured by PT (A) and aPTT (B) assay, respectively. Neither rivaroxaban nor DE affected orthotopic tumor growth as shown by weekly caliper measurements (C) and tumor weight (D) at the end of the experiment. Sensitive human-specific qPCR showed that rivaroxaban and DE did not significantly inhibit metastasis in lung (E) and liver (F); human cancer cells undetected in lungs of 33% (2/6) of DE-treated mice; human cancer cells detected in 60% (3/5), 0% (0/5), and 33% (2/6) of vehicle-treated, rivaroxaban-treated, and DE-treated mice, respectively. Two livers and lungs from mice that were not inoculated with cancer cells served as a negative control that remained negative for a human-specific GAPDH product. G, Gene expression was normalized to two housekeeping genes (GAPDH and ACTB) and shown as fold induction to Vehicle mice. N = 6 per experimental group; 1 mouse in vehicle and rivaroxaban group developed a tumor in peritoneum instead of in the fourth MFP, and were considered misinjected and excluded from further analyses. Two-tailed unpaired student *t*-test (A,B), 1-way ANOVA with Tukey's multiple comparison test (D,E,F), and 2-way ANOVA with Tukey's multiple comparison test (C) and 1-way ANOVA with Dunnett's test with additional original false discovery rate method of Benjamini and Hochberg method to correct for multiple testing of the 8 genes (G) were used for statistical evaluation. aPTT, activated partial thromboplastin time; PT, prothrombin time; qPCR, quantitative polymerase chain reaction.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs Vehicle

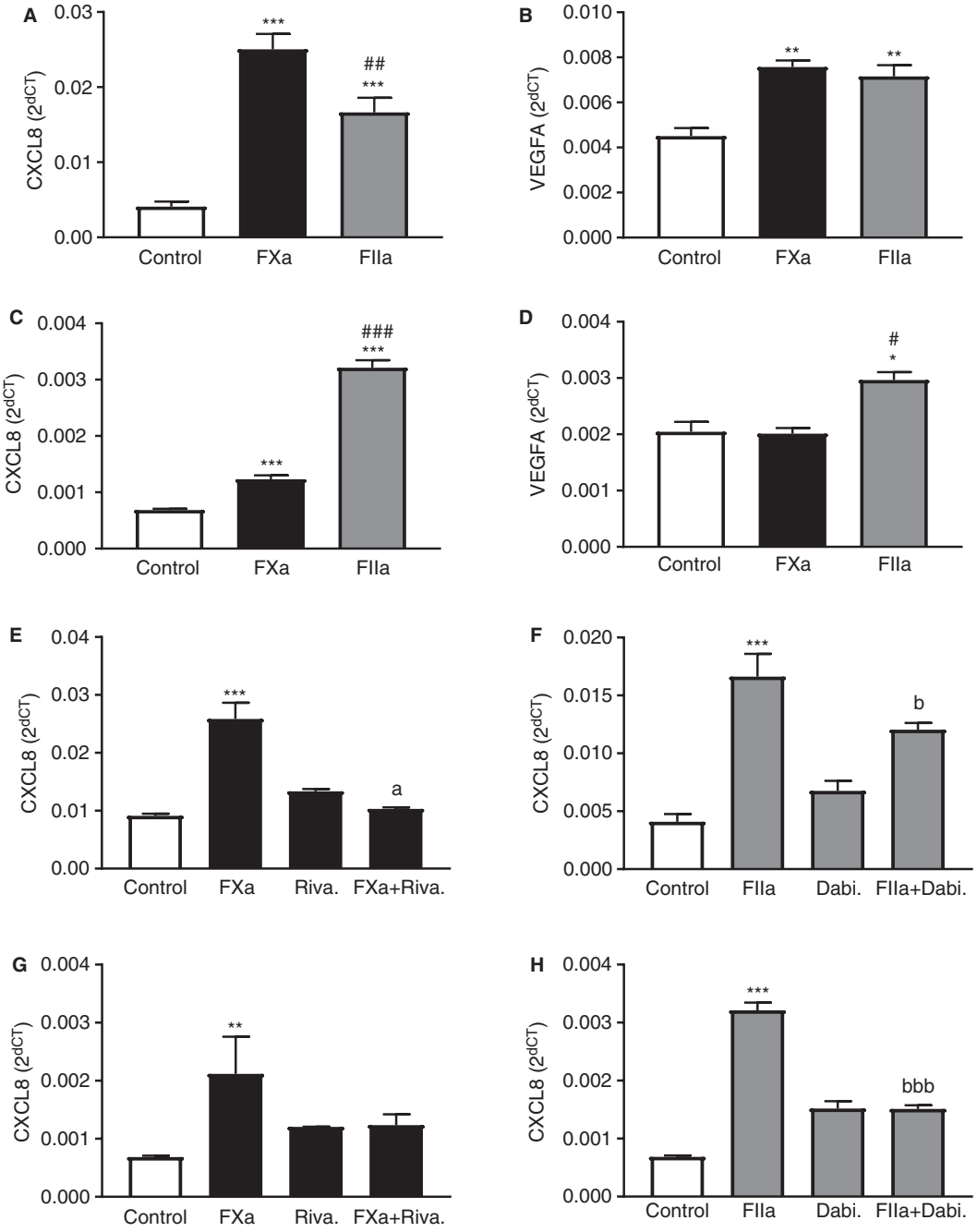

FIGURE 5 The coagulation factors FXa and thrombin induce target gene expression in MDA-MB-231 and HCC1806 cells as detected by qPCR. A, B, Stimulation of MDA-MB-231 cells with FXa (20 nmol/L) or FIIa (10 nmol/L) for 1 h increased CXCL8 (A) and VEGFA (B), mRNA expression levels. CXCL8 (C) and VEGFA (D) mRNA expression levels in HCC1806 cells stimulated with FXa (20 nmol/L) or FIIa (10 nmol/L) for 6 h. MDA-MB-231 cells were stimulated with FXa (2 nmol/L) and/or its inhibitor rivaroxaban (Riva., 1 μmol/L) (E) or stimulated for 1 h with FIIa (10 nmol/L) and/or its inhibitor dabigatran (Dabi., 1 μmol/L) (F). G, H, HCC1806 cells were stimulated with FXa (2 nmol/L) and/or its inhibitor rivaroxaban (1 μmol/L) (G) or stimulated for 6 h with FIIa (10 nmol/L) and/or its inhibitor dabigatran (1 μmol/L) (H). Gene expression was normalized to two housekeeping genes (GAPDH and ACTB) and calculated with the ΔCt method: $2^{[(Ct[GAPDH] + Ct[ACTB])/2)) - Ct[GOI]}$. Three independent experiments were performed, and a representative experiment is shown. For statistical evaluation, a 1-way ANOVA with Tukey's multiple comparison test was performed (A-H). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs Control, $^{\#}P < 0.05$ vs FXa, $^{\#\#}P < 0.01$ vs FXa, $^{\#\#\#}P < 0.001$ vs FXa; $^{a}P < 0.05$ vs FXa; $^{b}P < 0.05$ vs FIIa, $^{bbb}P < 0.001$ vs FIIa, thrombin; FXa, factor Xa; qPCR, quantitative polymerase chain reaction

lung and liver was found to be largely reduced in NOD-SCID mice when compared to NSG mice. Nonetheless, as observed before in NSG mice, rivaroxaban did not significantly affect orthotopic growth (Figure 2C,D) or metastasis formation in lungs or livers (Figure 2E,F) in NOD-SCID mice. Rivaroxaban treatment did not significantly affect gene expression in the primary tumor (Figure 2G).

Next, the effect of clinically relevant dosing of a LMWH was studied in the orthotopic model. To reduce the burden of daily s.c. injection with LMWH, we aimed at reducing the time frame of the in vivo experiment by using MDA-MB-231-LC cells, a more aggressive lung metastatic clone of the MDA-MB-231 cells. We studied the effects of rivaroxaban on tumor growth and metastasis in parallel. Coagulation parameters were significantly affected by LMWH and rivaroxaban treatment, but tumor growth and metastasis were unaffected (Figure 3, Figure S3A,B). The LMWH and rivaroxaban treatment did not significantly affect gene expression in the primary tumor (Figure 3G).

To extend our observations to another TNBC cell line, we used an orthotopic model with HCC1806 TNBC cells in a comparable experimental setup with NOD-SCID mice that were fed custom-made chow diet supplemented with rivaroxaban (1.0 mg/g chow diet) or DE (10 mg/g chow diet). Comparable to the earlier experiments, coagulation parameters were significantly affected by rivaroxaban and DE (Figure 4A,B) treatment. Whereas a small increase in tumor size was observed in rivaroxaban-treated mice at day 18 and day 21, no differences in tumor size and weight were observed at the end of the experiment (day 28) (Figure 4C,D). In the HCC1806 model, very low levels of cancer cells were detected in lung and liver as exemplified by the very low dCt values (Figure 4E,F). Nonetheless, metastatic cells in lungs were detected by qPCR in 100% of vehicle and rivaroxaban-treated mice and in 67% of DE-treated mice, and liver metastatic cells were detected in 60%, 0%, and 33% of vehicle, rivaroxaban-treated, and DE-treated mice, respectively (Figure 4E,F). Taken together, both rivaroxaban and DE did not significantly affect metastases in lung, liver, bone, or brain (Figure 4E,F, Figure S2C,D). Rivaroxaban and DE also did not significantly affect gene expression in the primary tumor of HCC1806 cells (Figure 4G). In none of the in vivo experiments was it observed that rivaroxaban or DE treatment affected body weight (Figure S4).

Subsequently, we aimed to exclude that the lack of effect of rivaroxaban and DE was due to diminished coagulation factor signaling in these cells. As expected, FXa and thrombin readily activated intracellular signaling in MDA-MB-231 and HCC1806 cells, as shown by induction of mRNA expression levels of the target genes *CXCL8* and *VEGFA* (Figure 5A-D). Moreover, FXa-induced and thrombin-induced *CXCL8* and *VEGFA* mRNA expression levels could be reduced by cotreatment with 1 μM rivaroxaban or dabigatran, respectively (Figure 5E-H).

Despite the induction of these downstream targets, FXa and thrombin were unable to induce migration of either MDA-MB-231 or HCC1806 cells in a live cell imaging-based assay in the presence of 0% or 10% FBS (Figure S5A,B). While MDA-MB-231 cells did not show any significant response to FBS exposure, the motility of HCC1806 cells was significantly enhanced in the presence of 10% FBS when compared to the 0% FBS condition. However, addition of either FXa or thrombin did not affect the migratory behavior of either TNBC cell line, even in serum-free conditions.

Using an MTS assay we observed that FXa and thrombin did not affect the number of viable MDA-MB-231 or HCC1806 cells in the presence of either 0% or 10% FBS (Figure S6). To study potential effects of FXa and thromin on breast cancer stem cells, clonogenic and mammosphere assays were performed. Factor Xa did not affect clonogenicity of either MDA-MB-231 or HCC1806 cells (Figure S7A,B). On the other hand, a relatively high concentration of thrombin (50 nmol/L) significantly inhibited the clonogenicity of MDA-MB-231 cells (Figure S7A). A comparable trend for inhibitory effects on clonogenicity by thrombin (50 nmol/L) was observed in HCC1806 cells (Figure S7B). Using the mammosphere assay, the size of HCC1806 mammospheres was also inhibited by 50 nmol/L thrombin (Figure S7D). Although the size of mammospheres was unaffected for FXa, the highest concentration used of FXa (20 nmol/L) inhibited mammosphere-forming efficiency (Figure S7C).

4 | DISCUSSION

Cancer patients with VTE, or at high risk for VTE, receive routine thromboprophylaxis. In preclinical cancer models, the clinically available anticoagulants LMWH and VKA have been shown to inhibit tumor growth and metastasis. This was at least in part mediated independently of its effects on coagulation.[21,27,47] A novel class of anticoagulants, the so-called DOACs, were demonstrated to reduce VTE recurrence effectively in patients without cancer. Recently, FXa-targeting DOACs (edoxaban and rivaroxaban) also reduced VTE recurrence in cancer patients.[36,37] To date, the potential anticancer effects of FXa-targeting DOACs have not been addressed in preclinical cancer models.

In this study, we have shown that both a FXa-targeting and a thrombin-targeting DOAC affected coagulation parameters as expected, but did not affect human breast cancer progression in NSG and NOD-SCID mice. Nevertheless, we cannot rule out that these two DOACs or the LMWH dalteparin used in our orthotopic models would have inhibited metastasis formation in an experimental metastasis model in which cancer cells are directly inoculated into the circulation.

While in patients with solid cancers, tumor cells may intravasate into the vasculature and metastasize at any time, peak LMWH plasma levels are only reached in the hours after LMWH injection.[48] The inhibitory effects of LMWHs on metastasis in the experimental metastasis models were found in an experimental setting in which LMWHs were injected directly before (0-4 h) or at the time of cancer cell inoculation.[18,20,22,23] Although mechanistically interesting, we believe that the orthotopic model used is clinically more relevant and we therefore refrained from using the experimental metastasis model.

In contrast to FXa-targeting DOACs, a few studies have addressed the role of thrombin-targeting DOACs in preclinical cancer models. In an orthotopic model using breast cancer cells of murine origin, DeFeo et al showed that DE treatment - started 1 day before orthotopic inoculation of cancer cells - inhibited cancer growth at primary and metastatic sites.[49] Tumor growth was not affected when DE treatment was started 16 days after orthotopic inoculation of breast cancer cells, but DE treatment potentiated the antitumor effects of the chemotherapeutic drug

cyclophosphamide in this setting, via reducing the number of TF-positive microparticles and decreasing the number of activated platelets.[50] A different picture emerged in a xenograft model using pancreatic cells of human origin, where DE did not affect cancer growth or metastasis, but increased CD31+ vessel density and proliferation, potentiating the effects of the chemotherapeutic drug gemcitabine.[51]

In line with the current notion in literature that NK cells play a prominent role in the control of metastasis,[52] we found that although tumor growth at the orthotopic site was slightly affected, the metastatic load in lungs and liver was largely reduced in NOD-SCID mice when compared to NSG mice. Nonetheless, we could not find antitumor effects of DOACs in either mouse strain. A limitation of our study is that - although NK cell proficient - NOD-SCID mice do not have a full immune competent system. On the other side, this allowed us to study effects of anticoagulants on clinically more relevant human cancer cells.

In the absence of randomized clinical trials that compare FXa-targeting DOACs head to head with thrombin-targeting DOACs, it remains speculative which coagulation factor would be a better target in cancer patients with VTE.[53,54] As the final effector of blood coagulation, converting fibrinogen to fibrin and exerting thrombin-mediated positive feedback loops, thrombin seems a logical primary target for an anticoagulation treatment. Moreover, thrombin also serves as a potent platelet agonist resulting in activated platelets that augment thrombin generation, whereas FXa has no effect on platelet activation.[55]

In the coagulation cascade, the intrinsic and extrinsic pathway converge at FX activation, where one molecule of FXa results in the generation of 1000 molecules of thrombin.[56] Therefore, it may well be proposed that FXa is a better target than thrombin.[57] This is further substantiated by clinical data that heparins with the highest anti-Xa:thrombin activity have been found to be more effective in patients with VTE.[58,59] Furthermore, the FXa inhibitor fondaparinux has superior antithrombotic potential and safety compared to the LMWH enoxaparin, which exerts both anti-Xa and antithrombin activity.[59]

Besides effects on coagulation, thrombin can also activate PAR1-signaling in cancer cells and platelets, exerting a variety of protumorigenic effects including induction of migration, invasion, cancer cell adhesion, and induction of metastatic potential in an experimental metastasis model.[60,61] Factor Xa can activate both PAR1 and PAR2 signaling, but its role in cancer is less clearly defined. While it has been shown that the TF-FVIIa-Xa complex induced migration by promoting phosphorylation of the p44/42 mitogen-activated protein kinase, Akt/protein kinase B, and mTOR pathway,[14,62] others demonstrated that FXa inhibited migration via PAR1-mediated Rho/ROCK and Src/FAK/paxillin-signalling in breast cancer cells.[63]

In the absence of randomized clinical trials, a network meta-analysis was conducted to compare the efficacy and safety of the DOACs rivaroxaban, apixaban, edoxaban, and dabigatran in the treatment of VTE.[31] All DOACs showed statistically similar reductions in the risk of VTE or VTE-related death. A significant reduction in number of bleedings was established with apixaban compared with all other DOACs, and with dabigatran compared with rivaroxaban and edoxaban, suggesting that the differences between these DOACs are not solely explained by the target (FXa or thrombin). How these data hold in cancer patients remains to be studied.

Taken together, we have shown that although effectively inhibiting coagulation, the DOACs rivaroxaban and DE did not affect orthotopic growth and metastasis of human TNBC. It remains to be investigated whether FXa-targeting and thrombin-targeting DOACs exert antitumorigenic effects in other types of cancer.

ACKNOWLEDGMENTS

This work was supported by grants from the Dutch Cancer Society (#2015-7594) and the World Wide Cancer Research (#15-1186).

DISCLOSURE OF CONFLICT OF INTEREST

The authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

J.T. Buijs designed the experiments, acquired and interpreted the data, and wrote the manuscript. E.H. Laghmani, R.F.P. van den Akker, C. Tieken, E.M. Vletter, K.M. van der Molen, and J. Crooijmans generated and critically interpreted data. C. Kroone generated data and critically revised the manuscript. S.E. Le Dévédec acquired and interpreted the data from the live cell imaging-based migration assay and critically revised the manuscript. G. van der Pluijm assisted in design of the study and revised the manuscript. H.H. Versteeg supervised and designed the study and critically revised the manuscript. All authors reviewed the manuscript and approved the final version.

ORCID

Jeroen T. Buijs https://orcid.org/0000-0001-5816-9739

Henri H. Versteeg https://orcid.org/0000-0003-4294-175X

REFERENCES

1. Blom JW, Doggen CJ, Osanto S, Rosendaal FR. Malignancies, prothrombotic mutations, and the risk of venous thrombosis. JAMA. 2005;293:715–22.
2. Sutherland DE, Weitz IC, Liebman HA. Thromboembolic complications of cancer: epidemiology, pathogenesis, diagnosis, and treatment. Am J Hematol. 2003;72:43–52.
3. Timp JF, Braekkan SK, Versteeg HH, Cannegieter SC. Epidemiology of cancer-associated venous thrombosis. Blood. 2013;122:1712–23.
4. Khorana AA, Francis CW, Culakova E, Kuderer NM, Lyman GH. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. J Thromb Haemost. 2007;5:632–4.

5. Chew HK, Wun T, Harvey D, Zhou H, White RH. Incidence of venous thromboembolism and its effect on survival among patients with common cancers. Arch Intern Med. 2006;166:458–64.
6. Lee AY, Levine MN, Baker RI, Bowden C, Kakkar AK, Prins M, et al. Low-molecular-weight heparin versus a coumarin for the prevention of recurrent venous thromboembolism in patients with cancer. N Engl J Med. 2003;349:146–53.
7. Kakkar AK, Levine MN, Kadziola Z, Lemoine NR, Low V, Patel HK, et al. Low molecular weight heparin, therapy with dalteparin, and survival in advanced cancer: the fragmin advanced malignancy outcome study (FAMOUS). J Clin Oncol. 2004;22:1944–8.
8. Klerk CP, Smorenburg SM, Otten HM, Lensing AW, Prins MH, Piovella F, et al. The effect of low molecular weight heparin on survival in patients with advanced malignancy. J Clin Oncol. 2005;23:2130–5.
9. Sideras K, Schaefer PL, Okuno SH, Sloan JA, Kutteh L, Fitch TR, et al. Low-molecular-weight heparin in patients with advanced cancer: a phase 3 clinical trial. Mayo Clin Proc. 2006;81:758–67.
10. Sanford D, Naidu A, Alizadeh N, Lazo-Langner A. The effect of low molecular weight heparin on survival in cancer patients: an updated systematic review and meta-analysis of randomized trials. J Thromb Haemost. 2014;12:1076–85.
11. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013;93:327–58.
12. Garnier D, Milsom C, Magnus N, Meehan B, Weitz J, Yu J, et al. Role of the tissue factor pathway in the biology of tumor initiating cells. Thromb Res. 2010;125(suppl 2):S44–50.
13. Hjortoe GM, Petersen LC, Albrektsen T, et al. Tissue factor-factor VIIa-specific up-regulation of IL-8 expression in MDA-MB-231 cells is mediated by PAR-2 and results in increased cell migration. Blood. 2004;103:3029–37.
14. Jiang X, Zhu S, Panetti TS, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex induces activation of the mTOR pathway which regulates migration of human breast cancer cells. Thromb Haemost. 2008;100:127–33.
15. Morris DR, Ding Y, Ricks TK, Gullapalli A, Wolfe BL, Trejo J. Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. Cancer Res. 2006;66:307–14.
16. Palumbo JS, Talmage KE, Massari JV, et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. Blood. 2005;105:178–85.
17. Palumbo JS, Talmage KE, Massari JV, et al. Tumor cell-associated tissue factor and circulating hemostatic factors cooperate to increase metastatic potential through natural killer cell-dependent and-independent mechanisms. Blood. 2007;110:133–41.
18. Amirkhosravi A, Mousa SA, Amaya M, Francis JL. Antimetastatic effect of tinzaparin, a low-molecular-weight heparin. J Thromb Haemost. 2003;1:1972–6.
19. Bereczky B, Gilly R, Raso E, Vago A, Timar J, Tovari J. Selective antimetastatic effect of heparins in preclinical human melanoma models is based on inhibition of migration and microvascular arrest. Clin Exp Metastasis. 2005;22:69–76.
20. HQ, Elkin M, Aingorn E, Ishai-Michaeli R, Stein CA, Vlodavsky I. Inhibition of heparanase activity and tumor metastasis by laminarin sulfate and synthetic phosphorothioate oligodeoxynucleotides. Int J Cancer. 1999;83:424–31.
21. Mousa SA, Linhardt R, Francis JL, Amirkhosravi A. Anti-metastatic effect of a non-anticoagulant low-molecular-weight heparin versus the standard low-molecular-weight heparin, enoxaparin. Thromb Haemost. 2006;96:816–21.
22. Pollari S, Kakonen RS, Mohammad KS, Rissanen JP, Halleen JM, Warri A, et al. Heparin-like polysaccharides reduce osteolytic bone destruction and tumor growth in a mouse model of breast cancer bone metastasis. Mol Cancer Res. 2012;10:597–604.
23. Stevenson JL, Choi SH, Varki A. Differential metastasis inhibition by clinically relevant levels of heparins–correlation with selectin inhibition, not antithrombotic activity. Clin Cancer Res. 2005;11:7003–11.
24. Bobek V, Boubelik M, Fiserova A, L'Uptovcova M, Vannucci L, Kacprzak G, et al. Anticoagulant drugs increase natural killer cell activity in lung cancer. Lung Cancer. 2005;47:215–23.
25. Smorenburg SM, Vink R, te Lintelo M, et al. In vivo treatment of rats with unfractionated heparin (UFH) or low molecular weight heparin (LMWH) does not affect experimentally induced colon carcinoma metastasis. Clin Exp Metastasis. 1999;17:451–6.
26. Harvey JR, Mellor P, Eldaly H, Lennard TW, Kirby JA, Ali S. Inhibition of CXCR4-mediated breast cancer metastasis: a potential role for heparinoids? Clin Cancer Res. 2007;13:1562–70.
27. Hostettler N, Naggi A, Torri G, et al. P-selectin- and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. FASEB J. 2007;21:3562–72.
28. Schlesinger M, Roblek M, Ortmann K, et al. The role of VLA-4 binding for experimental melanoma metastasis and its inhibition by heparin. Thromb Res. 2014;133:855–62.
29. Takahashi H, Ebihara S, Okazaki T, Asada M, Sasaki H, Yamaya M. A comparison of the effects of unfractionated heparin, dalteparin and danaparoid on vascular endothelial growth factor-induced tumour angiogenesis and heparanase activity. Br J Pharmacol. 2005;146:333–43.
30. Kirane A, Ludwig KF, Sorrelle N, et al. Warfarin blocks Gas6-mediated Axl activation required for pancreatic cancer epithelial plasticity and metastasis. Cancer Res. 2015;75:3699–705.
31. Cohen AT, Hamilton M, Mitchell SA, Phatak H, Liu X, Bird A, et al. Comparison of the novel oral anticoagulants apixaban, dabigatran, edoxaban, and rivaroxaban in the initial and long-term treatment and prevention of venous thromboembolism: systematic review and network meta-analysis. PLoS ONE. 2015;10:e0144856.
32. Perzborn E, Roehrig S, Straub A, Kubitza D, Misselwitz F. The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor. Nat Rev Drug Discov. 2011;10:61–75.
33. Buller HR, Decousus H, Grosso MA, et al. Edoxaban versus warfarin for the treatment of symptomatic venous thromboembolism. N Engl J Med. 2013;369:1406–15.
34. Buller HR, Prins MH, Lensin AW, et al. Oral rivaroxaban for the treatment of symptomatic pulmonary embolism. N Engl J Med. 2012;366:1287–97.
35. Lee AY, Peterson EA. Treatment of cancer-associated thrombosis. Blood. 2013;122:2310–7.
36. Raskob GE, van Es N, Verhamme P, et al. Edoxaban for the treatment of cancer-associated venous thromboembolism. N Engl J Med. 2018;378:615–24.
37. Young AM, Marshall A, Thirlwall J, et al. Comparison of an oral factor Xa inhibitor with low molecular weight heparin in patients with cancer with venous thromboembolism: results of a randomized trial (SELECT-D). J Clin Oncol. 2018;36:2017–23.
38. van Es N, Di Nisio M, Bleker SM, et al. Edoxaban for treatment of venous thromboembolism in patients with cancer. Rationale and design of the Hokusai VTE-cancer study. Thromb Haemost. 2015;114:1268–76.
39. Mahler Convenor M, Berard M, Feinstein R, et al. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab Anim. 2014;48:178–92.
40. Kocaturk B, Versteeg HH. Orthotopic injection of breast cancer cells into the mammary fat pad of mice to study tumor growth. J Vis Exp. 2015;96:51967.
41. van Roosmalen W, Le Devedec SE, Golani O, et al. Tumor cell migration screen identifies SRPK1 as breast cancer metastasis determinant. J Clin Invest. 2015;125:1648–64.

42. van Roosmalen W, Le Devedec SE, Zovko S, de Bont H, van de Water B. Functional screening with a live cell imaging-based random cell migration assay. Methods Mol Biol. 2011;769:435–48.
43. Sparkenbaugh EM, Chantrathammachart P, Mickelson J, et al. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. Blood. 2014;123:1747–56.
44. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood. 2002;100:3175–82.
45. Dewan MZ, Terunuma H, Takada M, et al. Role of natural killer cells in hormone-independent rapid tumor formation and spontaneous metastasis of breast cancer cells in vivo. Breast Cancer Res Treat. 2007;104:267–75.
46. Dewan MZ, Terunuma H, Ahmed S, et al. Natural killer cells in breast cancer cell growth and metastasis in SCID mice. Biomed Pharmacother. 2005;59(suppl 2):S375–9.
47. Stevenson JL, Varki A, Borsig L. Heparin attenuates metastasis mainly due to inhibition of P- and L-selectin, but non-anticoagulant heparins can have additional effects. Thromb Res. 2007;120(suppl 2):S107–11.
48. Pineo GF, Hull RD. Dalteparin: pharmacological properties and clinical efficacy in the prophylaxis and treatment of thromboembolic diseases. Eur J Med Res. 2004;9:215–24.
49. DeFeo K, Hayes C, Chernick M, Ryn JV, Gilmour SK. Use of dabigatran etexilate to reduce breast cancer progression. Cancer Biol Ther. 2010;10:1001–8.
50. Alexander ET, Minton AR, Hayes CS, Goss A, Van Ryn J, Gilmour SK. Thrombin inhibition and cyclophosphamide synergistically block tumor progression and metastasis. Cancer Biol Ther. 2015;16:1802–11.
51. Shi K, Damhofer H, Daalhuisen J, Ten Brink M, Richel DJ, Spek CA. Dabigatran potentiates gemcitabine-induced growth inhibition of pancreatic cancer in mice. Mol Med. 2017;23:13–23.
52. Lopez-Soto A, Gonzalez S, Smyth MJ, Galluzzi L. Control of Metastasis by NK Cells. Cancer Cell. 2017;32:135–54.
53. Ansell J. Factor Xa or thrombin: is factor Xa a better target? J Thromb Haemost. 2007;5(suppl 1):60–4.
54. Weitz JI. Factor Xa or thrombin: is thrombin a better target? J Thromb Haemost. 2007;5(suppl 1):65–7.
55. Ieko M, Tarumi T, Takeda M, Naito S, Nakabayashi T, Koike T. Synthetic selective inhibitors of coagulation factor Xa strongly inhibit thrombin generation without affecting initial thrombin forming time necessary for platelet activation in hemostasis. J Thromb Haemost. 2004;2:612–8.
56. Mann KG, Brummel K, Butenas S. What is all that thrombin for? J Thromb Haemost. 2003;1:1504–14.
57. Yin ET, Wessler S. Heparin-accelerated inhibition of activated factor X by its natural plasma inhibitor. Biochim Biophys Acta. 1970;201:387–90.
58. Howard AW, Aaron SD. Low molecular weight heparin decreases proximal and distal deep venous thrombosis following total knee arthroplasty. A meta-analysis of randomized trials. Thromb Haemost. 1998;79:902–6.
59. Turpie AG, Bauer KA, Eriksson BI, Lassen MR. Fondaparinux vs enoxaparin for the prevention of venous thromboembolism in major orthopedic surgery: a meta-analysis of 4 randomized double-blind studies. Arch Intern Med. 2002;162:1833–40.
60. Franchini M, Mannucci PM. Thrombin and cancer: from molecular basis to therapeutic implications. Semin Thromb Hemost. 2012;38:95–101.
61. Nierodzik ML, Klepfish A, Karpatkin S. Role of platelets, thrombin, integrin IIb-IIIa, fibronectin and von Willebrand factor on tumor adhesion in vitro and metastasis in vivo. Thromb Haemost. 1995;74:282–90.
62. Jiang X, Bailly MA, Panetti TS, Cappello M, Konigsberg WH, Bromberg ME. Formation of tissue factor-factor VIIa-factor Xa complex promotes cellular signaling and migration of human breast cancer cells. J Thromb Haemost. 2004;2:93–101.
63. Borensztajn K, Peppelenbosch MP, Spek CA. Coagulation factor Xa inhibits cancer cell migration via LIMK1-mediated cofilin inactivation. Thromb Res. 2010;125:e323–8.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Buijs JT, Laghmani EH, van den Akker RFP, et al. The direct oral anticoagulants rivaroxaban and dabigatran do not inhibit orthotopic growth and metastasis of human breast cancer in mice. *J Thromb Haemost*. 2019;17:951–963. https://doi.org/10.1111/jth.14443