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Dabigatran reduces thrombin-induced platelet aggregation and activation in a dose-dependent manner

Pernille Just Vinholt[1,3,4] · Christian Nielsen[2] · Anna Cecilia Söderström[1,4] · Axel Brandes[5] · Mads Nybo[1]

Abstract Dabigatran is an oral anticoagulant and a reversible inhibitor of thrombin. Further, dabigatran might affect platelet function through a direct effect on platelet thrombin receptors. The aim was to investigate the effect of dabigatran on platelet activation and platelet aggregation. Healthy donor blood was incubated with dabigatran 0, 50, 500 ng/mL, corresponding to the therapeutic range of dabigatran peak plasma concentrations, and 10,000 ng/mL comprising a supra-therapeutic dabigatran plasma level. Platelet aggregation was tested with 96-well aggregometry. Flow cytometry was used to test platelet activation and platelet thrombin receptor expression (SPAN-12 and WEDE-15 expression). Agonists were thrombin, thrombin receptor-activating peptide, protease-activated receptor-4 agonist, collagen, collagen-related peptide, arachidonic acid, and adenosine diphosphate. All concentrations of dabigatran fully inhibited platelet aggregation for thrombin up to 2 IU/mL, while dabigatran did not affect platelet aggregation by other agonists. Platelet activation (percentage of platelets positive for activated GPIIb/IIIa, CD63, P-selectin) was reduced after thrombin stimulation in samples with dabigatran levels ≥500 ng/mL. After stimulation with thrombin, the percentage of activated GPIIb/IIIa-positive platelets was 99.8 ± 0.2% without dabigatran, 14.7 ± 4.7% with 500 ng/mL dabigatran, and 4.2 ± 0.2% with 10,000 ng/mL dabigatran, both $p < 0.001$ when compared to samples without dabigatran. Also, the receptor expression of GPIIb/IIIa, CD63, and P-selectin were reduced after dabigatran treatment. The expression of thrombin receptors was reduced at dabigatran on ≥ 500 ng/mL. In conclusion, dabigatran exclusively inhibits thrombin-induced platelet activation and aggregation with a dose-dependent response. Platelet stimulation with other agonists was not affected by dabigatran.

Keywords Thrombin inhibitor · Platelet activation · Platelet aggregation

Introduction

Dabigatran is an orally administered anticoagulant for prevention of thromboembolism in patients with atrial fibrillation and for prevention and treatment of venous thromboembolism [1, 2]. It is commonly administered at fixed doses of 110 or 150 mg twice daily [1]. As dabigatran is a direct thrombin inhibitor, it has effect on the secondary haemostasis [3]. As the main adverse event of dabigatran treatment is bleeding [1], it is relevant whether dabigatran has additional effects on platelet function. If so, it could affect the bleeding risk and influence the choice of treatment based on the patient risk profile. The effect of dabigatran on platelet activation has not been studied, while previous studies showed that dabigatran inhibits tissue factor and thrombin-induced platelet aggregation when testing effects of dabigatran in vitro [3, 4]. It could be an indirect effect due to

✉ Pernille Just Vinholt
pernille.vinholt@rsyd.dk

1 Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark

2 Department of Clinical Immunology, Odense University Hospital, Odense, Denmark

3 Department of Clinical Immunology and Biochemistry, Vejle Hospital, Kabbeltoft 25, 7100 Vejle, Denmark

4 OPEN Odense Patient Data Explorative Network, Clinical Institute, University of Southern Denmark, Odense, Denmark

5 Department of Cardiology, Odense University Hospital, Odense, Denmark

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the binding of dabigatran to generated thrombin which then prevents thrombin from stimulating platelets. Platelets express two thrombin receptors, protease-activated receptor (PAR)-1 and PAR-4, which are both stimulated with thrombin. In vitro PAR receptors can be selectively stimulated and studied with different peptides [5]. The effect of dabigatran on the PAR-4 receptor has not be studied, while findings are divergent on the effect of dabigatran on the PAR-1 receptor [4, 6–9].

The aim of the present study was to determine the effect of dabigatran on platelet activation and platelet aggregation.

Materials and methods

Participants and sample preparation

Participants were healthy individuals (blood donors) recruited from the blood bank at Department of Immunology at Odense University Hospital after informed consent. All patients gave written informed consent. The study was approved by the Regional Scientific Ethical Committees of Southern Denmark (s-20140186) and the Danish Data Protection Agency (2008-58-0035) and conducted in accordance with the guidelines of the Declaration of Helsinki.

Blood was drawn by venipuncture of a peripheral vein into 3.2% (109 mM) trisodium citrate anticoagulated blood tubes (Becton Dickinson, New Jersey, USA). Blood was centrifuged at 200×*g* for 10 min to obtain platelet-rich plasma (PRP). The remaining sample was re-centrifuged at 10,000×*g* for 10 min and platelet-poor plasma (PPP) collected. When isolating platelets, PRP was mixed with acid-citrate-dextrose solution (10% volume/volume) and prostaglandin E1 (Alprostadil 0.9 µM, Tocris, Bristol, United Kingdom) to prevent platelet activation. The PRP was then centrifuged 1000×*g* for 10 min during which platelets were pelleted. The platelet pellet was washed and suspended in dilution buffer (NaCl 134 mM, KCL 2.9 mM, $MgCl_2$ 1 mM, glucose 5.6 mM, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 20 mM, pH 7.4).

Dabigatran (BIBR 953, Selleckchem, Houston, TX, USA), which is the active metabolite of the drug, was suspended in dimethyl sulfoxide (DMSO) and further diluted in phosphate buffered saline (PBS). Whole blood, PRP or isolated platelets, were incubated with DMSO, 50, 500 or 10,000 ng/mL dabigatran for 30 min at 37 °C before used in tests [10].

Platelet activation by flow cytometry

Five microlitres whole blood was incubated for 10 min at room temperature in dilution buffer with 1 g/L bovine serum albumin and fluorophore-conjugated monoclonal antibodies (mAbs): phycoerythrin (PE)-conjugated glycoprotein(GP) Ib (CD42b, Clone HIP1) and allophycocyanin (APC)-conjugated P-selectin (CD62p, Clone Psel.KO2.3) (both eBioscience, San Diego, CA, USA); PE-cyanine 7 (PE-Cy7) granulophysin (CD63, clone H5C6) and fluorescein isothiocyanate (FITC)-conjugated PAC1 (both Becton Dickinson Bioscience, San Jose, CA, USA). The PAC1 mAb binds only to activated GPIIb/IIIa. In addition, either agonist or dilution buffer was added to a final volume of 65 µL. Final concentrations of agonists were 12.8 µM adenosine diphosphate (ADP; Sigma-Aldrich, St. Louis, Missouri, USA), 10 µM thrombin receptor-activating peptide (TRAP; SFLLRN, JPT Peptide Technologies GmbH, Berlin, Germany), 1.1 µg/mL collagen-related peptide (Dr. Richard W. Farndale, University of Cambridge, United Kingdom), 100 µM PAR-4 (AYPGKF-NH_2), 1–2 IU/mL thrombin with 1 mmol/L Gly-Pro-Arg-Pro [11]. Thrombin (T6884) was from Sigma Aldrich (St. Louis, Missouri, USA) while PAR-4 and Gly-Pro-Arg-Pro was from Bachem (Bubendorf, Switzerland). A negative sample was incubated with mAbs against CD63, CD42b and PAC1, and with an anti-P-selectin matched APC-conjugated isotype control (eBioscience, San Diego, CA, USA); EDTA (10 mM) was added to inhibit specific binding of PAC1 and CD63 mAbs [12]. Three samples were made per agonist, except for thrombin where nine samples were tested. Triplicates were conducted. Incubation was stopped by the addition of fixation buffer. Samples were acquired on the FACSCanto II (Becton Dickinson, Franklin Lakes, New Jersey, USA) and processed using Kaluza software 1.3 (Beckman Coulter, California, USA). Platelets were gated based on GPIb expression and scatter pattern. The results were expressed as mean fluorescence intensities (MFI) or, in the platelet reactivity assay also as the percentage of platelets positive for P-selectin, CD63 and/or activated GPIIb/IIIa when compared to the negative sample [12].

The PAR-1 receptor expression was evaluated using SPAN-12 and WEDE-15 antibodies (Beckman Coulter, Marseille, France). One part whole blood was mixed with nine parts of PBS. Twenty microliters of this dilution were incubated with 10 µL of PE-labelled anti-thrombin receptor antibody for 15 min at room temperature in the dark before addition of fixation buffer. The sample was then acquired on the flow cytometer [13]. The antibody comprised either SPAN-12 (8.3 µg/mL final concentration) or WEDE-15 (8.3 µg/mL final concentration) [13]. SPAN-12 recognizes an epitope that is lost when thrombin cleaves the receptor, while WEDE-15 recognizes epitopes on both cleaved and uncleaved receptors [14].

96-well aggregometry

Half-area 96-well microtitre plates (Greiner Bio-One, Stonehouse, Gloucestershire, United Kingdom) were coated with 5 µL PBS with or without agonist. Plates were sealed and stored on −80 °C until used (Vinholt et al., under review). The final concentration of platelet agonist was 0.5 mM arachidonic acid, 10 µM TRAP, 6.4 µg/mL collagen Type 1, 6.4 µM ADP, 100 µM PAR-4 agonist or 1–2 IU/mL thrombin. The same agonists were used as in the flow cytometric assays with few exceptions; collagen and arachidonic acid were from Roche Diagnostics (Mannheim, Germany) and collagen from Chrono-Log (Havertown, PA, USA) was also used. Ten samples were tested at each setting.

For testing platelet aggregation, 45 µL of PRP was added to wells with agonists. As reference, 45 µL of PRP or PPP was added to wells with PBS. Plates were applied into Victor X5 (Perkin Elmer, Turku, Finland), shaken (900 rpm, 10 min at 37 °C). Thereafter absorbance was measured and reported as optical density (OD). Platelet aggregation was calculated as $100\% \times (OD_{PRP} - OD_{sample})/(OD_{PRP} - OD_{PPP})$ (Vinholt et al., under review).

Statistics

Continuous results are reported as means and standard deviations. Groups were compared with Student's t test. Statistical procedures were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). P values were two-sided.

Results

Platelet activation was reduced for samples stimulated with thrombin (Fig. 1). The percentage of platelets positive for activated GPIIb/IIIa, CD63 and P-selectin after stimulation by thrombin was significantly lower in samples with dabigatran ≥ 500 ng/mL than without dabigatran, while the reduction was insignificant for samples with 50 ng/mL dabigatran. After thrombin stimulation, the percentage of GPIIb/IIIa-positive platelets was 99.8 ± 0.2% for samples without dabigatran, 14.7 ± 4.7% for samples with 500 ng/mL dabigatran, and 4.2 ± 0.2% for samples with 10,000 ng/mL dabigatran, both $p < 0.001$ when compared to samples without dabigatran. The expression of activated GPIIb/IIIa was reduced at all dabigatran concentrations, ($p < 0.05$); 20.5 ± 1.4 MFI in samples without dabigatran, 8.8 ± 7.7 MFI for 50 ng/mL, 1.5 ± 0.1 MFI for 500 ng/mL, and 1.3 ± 0.1 MFI for 10,000 ng/mL dabigatran.

After thrombin stimulation, the percentage of CD63-positive platelets was 73.8 ± 0.6% for samples without dabigatran and 1.7 ± 0.9% for samples with 500 ng/mL dabigatran and 0.2 ± 0.1% for samples with 10,000 ng/mL dabigatran, both $p < 0.001$ when compared to samples without dabigatran. The expression of CD63 after thrombin stimulation was significantly reduced at higher concentrations of dabigatran 500 − 10,000 ng/mL compared with samples without dabigatran, $p < 0.05$; 14.0 ± 1.2 MFI in samples without dabigatran, 12.3 ± 1.0 MFI for 50 ng/mL, 10.3 ± 0.8 MFI for 500 ng/mL, and 9.3 ± 1.7 MFI for 10,000 ng/mL dabigatran.

After thrombin stimulation, the percentage of P-selectin-positive platelets was 99.1 ± 0.1% for samples without dabigatran and 7.7 ± 2.8% for samples with 500 ng/mL dabigatran and 2.1 ± 0.3% for samples with 10,000 ng/mL dabigatran, both $p < 0.001$ when compared to samples without dabigatran. P-selectin-expression after thrombin stimulation was 56.4 ± 2.0 MFI in samples without dabigatran, 29.2 ± 1.7 MFI for 50 ng/mL, 7.0 ± 1.5 MFI for 500 ng/mL, and 4.4 ± 0.6 MFI for 10,000 ng/mL dabigatran. The

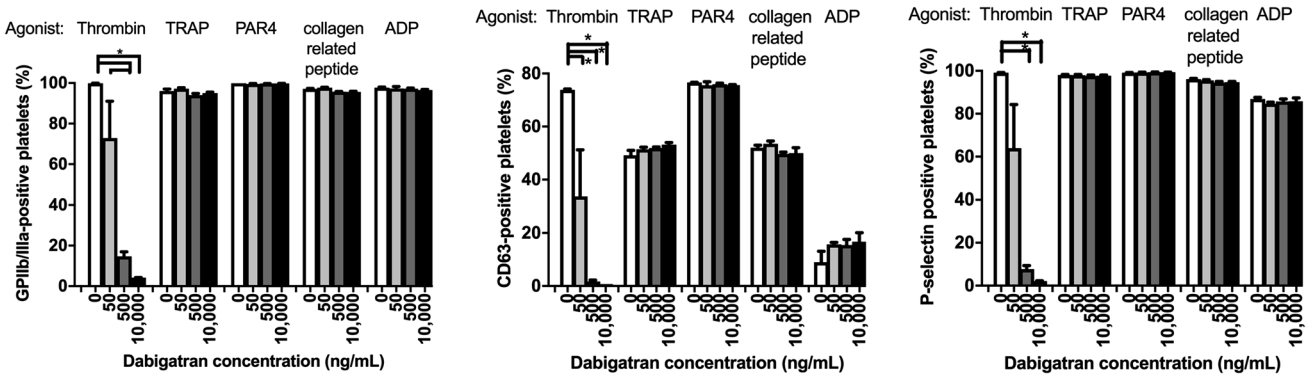

Fig. 1 The effect of dabigatran on platelet activation. Samples from healthy donors were spiked with dabigatran (0–10,000 ng/mL). Platelet activation was tested with flow cytometry, n = 9. *Graphs* show the percentage of activated glycoprotein (GP) IIb/IIIa, CD63 and P-selectin after stimulation with different agonists. *TRAP* thrombin receptor-activating peptide, *ADP* adenosine diphosphate, *PAR-4* agonist, protease-activated receptor 4 (AYPGKF-NH_2). Results are means ± standard deviations. Comparisons were made with t test, *$p < 0.05$

P-selectin-expression after thrombin stimulation was significantly reduced in all samples with dabigatran, ($p < 0.05$).

There was no effect of dabigatran in any concentration on the expression levels of the investigated platelet activation markers in samples stimulated with other agonists (collagen-related peptide, ADP, TRAP, or PAR-4).

WEDE-15 expression was lower in samples with ≥500 ng/mL dabigatran than in samples without dabigatran (Fig. 2). SPAN-12 expression was not reduced after incubation with 10,000 ng/mL dabigatran compared with samples without dabigatran.

Dabigatran did not affect platelet aggregation after stimulation with TRAP, PAR-4, arachidonic acid, collagen or ADP (Fig. 3). After addition of 1–2 IU/mL thrombin, mean platelet aggregation was 84% in samples without dabigatran and <6% at all dabigatran concentrations, $p < 0.05$ compared to samples without dabigatran. Screening with other agonists in various concentrations, did not reveal any clear dose-dependent effects of dabigatran on platelet aggregation responses (Fig. 4).

Discussion

We showed that dabigatran inhibits thrombin-induced platelet activation and platelet aggregation in a dose-dependent manner, but had no effect on other platelet activation pathways.

The tested dabigatran concentrations corresponded to the 2.5–97.5th percentile of the peak plasma dabigatran level determined by LC-MS after 150 mg twice daily

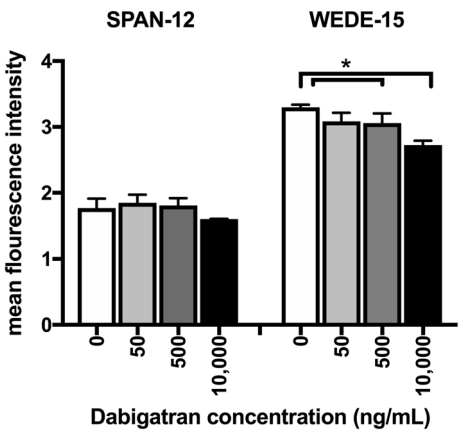

Fig. 2 The effect of dabigatran on protease-activated receptor-1 thrombin receptor expression. Samples from healthy donors were spiked with dabigatran (0–10,000 ng/mL). The expression of thrombin receptor was tested with flow cytometry, n = 3. The SPAN12 antibody recognizes an epitope that is lost when thrombin cleaves the receptor, while WEDE15 antibody recognizes epitopes cleaved and uncleaved receptor. Results are means ± standard deviations. Comparisons were made with t test, *$p < 0.05$

administration in patients with atrial fibrillation [15]. Moreover, a supra-therapeutic concentration of dabigatran (10,000 ng/mL) was tested. It is relevant, as dabigatran can accumulate, e.g. in elderly patients with kidney failure and might cause unfavorable effects such as higher bleeding risk in these patients. Dabigatran concentrations as high as 5600 ng/mL has been found [16], and was reported to have fatal outcome due to bleeding.

Thrombin is the strongest known platelet agonist and is generated at sites of vessel injury as a response to induced secondary haemostasis [17]. Overall, the present study confirmed that thrombin-induced platelet aggregation is reduced in all tested dosages of dabigatran. We propose that it is an indirect effect where dabigatran inhibits thrombin before it can act on platelets. In agreement, dabigatran had no effect on platelet aggregation when platelet aggregation was induced with peptides that recognize epitopes on the platelet thrombin receptors PAR-1 and PAR-4.

When thrombin is used as agonist, platelet isolation is required to avoid fibrin polymerization as fibrin polymerization increase absorbance in the samples and thus affect platelet aggregation results [11]. With addition of 2 IU/mL thrombin, which is approximately twice the normal plasma reserve and thus correspond to induced secondary haemostasis, platelet aggregation was absent regardless of the dabigatran concentration (50 − 10,000 ng/mL). This indicates that both the intrinsic thrombin pool and generated thrombin would be inhibited by the excess pool of free dabigatran [3]. This is a relevant antithrombotic mechanism, but potentially increases bleeding risk as it inhibits platelet response in case of vessel lesions.

Previous studies comprised primarily in vitro studies with spiked samples from healthy individuals with concentrations of dabigatran up to 2000 ng/mL. The studies only evaluated platelet aggregation and showed that dabigatran reduced tissue-factor and thrombin-induced platelet aggregation [3, 6], but dabigatran had no effect on platelet aggregation when healthy donor platelets were stimulated with ADP, arachidonic acid, TRAP (PAR-1 agonist) or collagen [3, 4, 6]. The present study adds that dabigatran up to 10,000 ng/mL do not affect TRAP, PAR-4, ADP, arachidonic acid, or collagen-induced platelet aggregation. We found a slight decline in expression of PAR-1 receptors with increasing dabigatran concentrations which did not translate into reduced platelet aggregation after stimulation of the receptor with TRAP. We add that nor PAR-4 induced platelet aggregation is inhibited by dabigatran. Previously, one study evaluated platelet function in platelets before and after starting dabigatran treatment found a rise in TRAP-induced platelet aggregation in patients after starting dabigatran. The explanation for this is not clear and the change was minor. The lack of effect on arachidonic and ADP-induced platelet aggregations indicates that dabigatran will

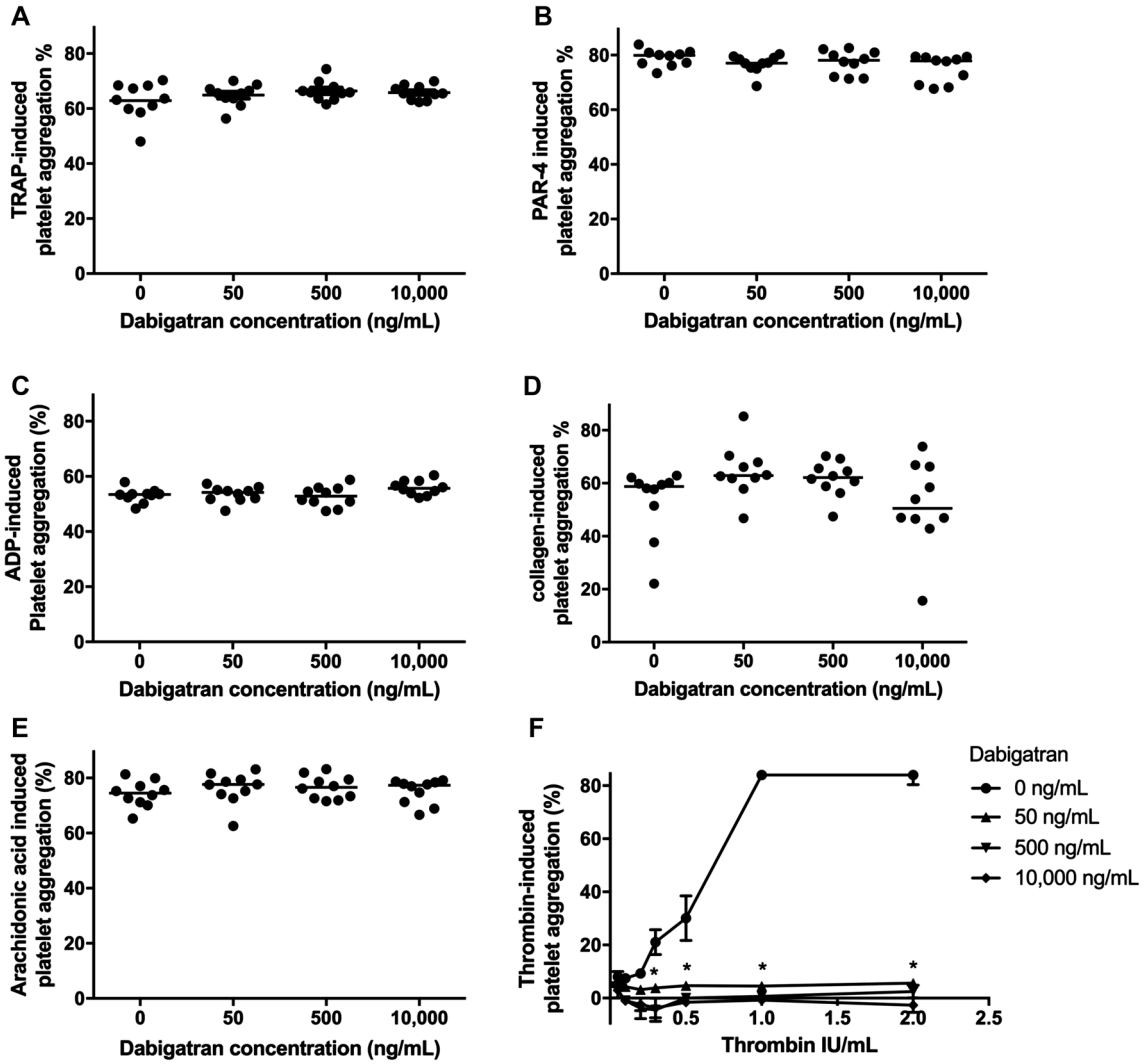

Fig. 3 The effect of dabigatran on platelet aggregation. Samples from healthy donors, n = 10, were spiked with dabigatran (0–10,000 ng/mL). Platelet aggregation was tested with 96-well platelet aggregometry. Platelet-rich plasma was used for experiments, except for thrombin-induced platelet aggregation, where isolated platelets were used. In graph **a**–**e**, *dots* are single measurements and *lines* reflect means. In graph **f**, *dots* are means and *bars* reflects standard deviations. *TRAP* thrombin receptor-activating peptide, *ADP* adenosine diphosphate, *PAR-4 agonist* protease-activated receptor 4 (AYPGKF–NH_2). Comparisons were made with t test, *$p < 0.05$

not influence the antiplatelet function of aspirin or ADP receptor agonists as e.g. clopidogrel [18].

We evaluated the effect of dabigatran on platelet activation with flow cytometry. We investigated both the percentage of platelets that were positive for each platelet activation marker and the expression level of each marker. It revealed that at dabigatran plasma concentration on 50 ng/mL (lower limit for therapeutic range), platelet activation capacity after thrombin stimulation was partially preserved in terms of the ability to activate the fibrinogen receptor and cause dense and alpha granule release. At dabigatran concentration on 500 ng/mL (upper limit for therapeutic range) platelet activation was significantly reduced. In agreement, the expression of thrombin receptors was reduced at dabigatran on ≥500 ng/mL which might contribute to the lower platelet activation capacity. In contrast, platelet aggregation after thrombin stimulation was inhibited at all dabigatran concentrations. It could reflect that the threshold for platelet stimulation is lower in whole blood than for isolated platelets in vitro. Our findings indicate that thrombin can cause platelet granule release at low dabigatran level of 50 ng/mL. It might suggest that thrombin generated or released at vessel injury to some extent overcome the inhibitory effect of dabigatran and result in granule release when dabigatran concentration is low within the therapeutic range. The release of granules could amplify platelet aggregation by other agonists due to the content of e.g. ADP in granules. If so, it may translate into variation

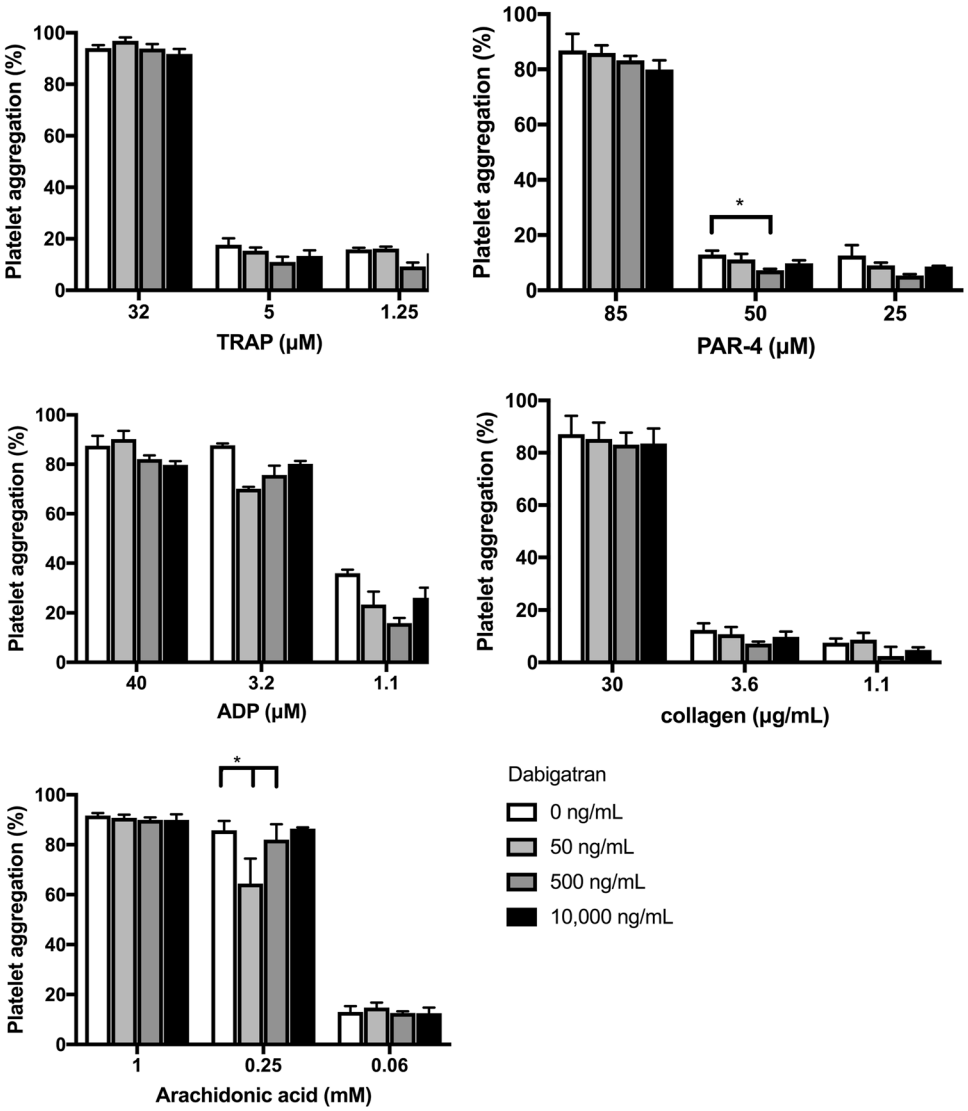

Fig. 4 The effect of dabigatran on platelet aggregation for high or low agonist concentrations. Samples from healthy donors were spiked with dabigatran in three concentrations (50–10,000 ng/mL). Platelet aggregation in platelet-rich plasma was tested with 96-well platelet aggregometry, n = 3. *TRAP* thrombin receptor-activating peptide, *ADP* adenosine diphosphate, *PAR-4 agonist* protease-activated receptor 4 (AYPGKF–NH_2). Results are means ± standard deviations Comparisons were made with t test, *p < 0.05

in bleeding risk. Further investigations into this mechanism would be of interest, as variation in bleeding risk within the therapeutic plasma dabigatran concentration was observed in the RE-LY study [19].

It is a strength of the present study that clinically relevant dabigatran doses were tested. Another strength is that we extensively studied multiple platelet activation pathways and addressed both platelet activation and platelet aggregation. It is a limitation that spiked blood samples were used as pharmacokinetics variability and any in vivo effects on receptor expression levels after long-term treatment will not be revealed. However, it allows for direct comparisons of the effect of dabigatran plasma concentrations on platelet function without any confounding factors. Dabigatran is a prodrug and we only evaluated the effect of the main active metabolite. If any unknown in vivo processing influences the effect of dabigatran, it would not be detected in our study.

In a clinical context, the European Medicine Agency stated that: "Dabigatran concentration under 48 ng/mL is equivalent to elimination of at least 75% of dabigatran and should be recommended before special intervention such as surgery" [20]. It corresponds to the lower limit for the therapeutic plasma dabigatran concentration. However, our study proposes that haemostasis is inhibited even at this dabigatran plasma concentration, which could influence the bleeding risk during invasive procedures.

In conclusion, dabigatran inhibited platelet activation at concentrations ≥500 ng/mL and inhibited thrombin induced platelet aggregation at all tested dabigatran concentrations (50–10,000 ng/mL).

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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