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ORIGINAL ARTICLE

Effects of rivaroxaban, acetylsalicylic acid and clopidogrel as monotherapy and in combination in a porcine model of stent thrombosis

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Summary. *Background:* Despite standard dual antiplatelet therapy (DAT) (acetylsalicylic acid [ASA] and clopidogrel), there is a ≥ 1.4% incidence of in-stent thrombosis in patients with acute coronary syndrome. Factor Xa inhibitors are being investigated for secondary prevention after acute coronary syndrome. *Objective:* To study the antithrombotic effects of the FXa inhibitor rivaroxaban alone or in combination with DAT. *Methods:* Bare metal stents (12 per animal, three per intervention period) were deployed in a porcine ex vivo arteriovenous shunt and exposed to flowing arterial blood (shear rate: 1500 s^{-1}). In-stent thrombus formation was analyzed under different treatments: vehicle (n = 7 animals); intravenous (i.v.) rivaroxaban (0.11, 0.33, and 1.0 μg kg^{-1} min^{-1}) (n = 8); rivaroxaban + ASA (1.0 mg kg^{-1} i.v.) (n = 6); rivaroxaban + ASA (1.0 mg kg^{-1} i.v.) + clopidogrel (0.5 mg kg^{-1} i.v.) (n = 7); and ASA (1.0 mg kg^{-1} i.v.) + clopidogrel (0.5 mg kg^{-1} i.v.) (n = 6). *Results:* Rivaroxaban dose-dependently reduced stent thrombus weight by ≤ 66% vs. vehicle (P < 0.05, all doses). Rivaroxaban + ASA further reduced thrombus weight vs. vehicle (86% at the highest rivaroxaban dose; P < 0.001). DAT reduced thrombus weight by ≤ 79%. However, rivaroxaban + ASA + clopidogrel almost completely abolished in-stent thrombus formation (98% reduction vs. vehicle at the highest rivaroxaban dose; P < 0.001). *Conclusions:* Our data on the inhibitory effect of rivaroxaban alone or with DAT are consistent with the ATLAS 2 trial findings, and support its potential use for preventing stent thrombosis after stent deployment.

Keywords: aspirin, clopidogrel, rivaroxaban, stent thrombosis.

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Introduction

Stent deployment is becoming the treatment of choice for coronary occlusions in patients with acute coronary syndrome (ACS). Unfortunately, however, the benefits are partially reduced by the incidence of in-stent thrombosis (IST).

The incidence of IST varies across studies and degrees of ACS severity. According to data from the prospective, randomized Acute Catheterization and Urgent Intervention Triage Strategy (ACUITY) trial, the incidence of definite or probable stent thrombosis within 30 days in patients with ACS still remained at 1.4% [1]. Higher incidence rates of up to 5.2% have been observed in patients with ST-segment elevation myocardial infarction ACS, according to other studies [2,3]. Furthermore, the rate of late stent thrombosis is four-fold to five-fold higher for drug-eluting stents than for bare metal stents, with thrombosis occurring approximately 15.5–18 months after stent placement [4]. It is of note that, despite the reduced rate of stent thrombosis, its presentation is usually fatal.

Thrombosis, either systemic or stent-mediated, plays a major role in the pathogenesis of IST. Current guidelines recommend the long-term administration of dual antiplatelet therapy (DAT) – a $P2Y_{12}$ antagonist (clopidogrel, prasugrel, or ticagrelor) and the cyclooxygenase inhibitor acetylsalicylic acid (ASA) [5]. In-stent thrombi have significant proportions of both platelets and fibrin [6–8]. It therefore seems rational to hypothesize that an antithrombotic approach combining both anticoagulant and antiplatelet agents should further reduce the risk of IST.

Factor Xa is present at the point of convergence of both the intrinsic and tissue factor (extrinsic) coagulation pathways, and inhibition of single downstream coagulation factors is becoming the new therapeutic target in the prevention and treatment of atherothrombotic events. The FXa inhibitor rivaroxaban has yielded promising results from phase II and III trials for secondary event prevention in ACS [9,10]. The recent phase III ATLAS ACS 2 TIMI 51 trial has shown the benefits of the rivaroxaban and antiplatelet combination in preventing

recurrent events in patients with ACS, and provides evidence of a reduction in the risk of stent thrombosis with rivaroxaban [10]. Rivaroxaban is a first-in-class, oral, direct FXa inhibitor [11]. Our aim was therefore to demonstrate the superior antithrombotic activity of the combination of dual standard antiplatelet therapy with a specific inhibitor of FXa in the prevention of IST.

Materials and methods

Experimental model and hemodynamics

All studies were performed on female anesthetized Göttingen minipigs (Ellegaard, Dalmose, Denmark) (n = 34; body weight, 27.8 ± 3.6 kg). Animals were initially sedated with ketamine hydrochloride (subcutaneous, 30 mg kg^{-1}; Ketavet; Pfizer, Berlin, Germany). Anesthesia was initiated with intravenous (i.v.) sodium thiopental (17 mg kg^{-1} Trapanal; Nycomed, Konstanz, Germany), and maintained with inhaled 2% enflurane (Shanghai FWD Chemicals, Shanghai, China) following endotracheal intubation. Animals were artificially ventilated at a constant volume (Sulla 808 V-D; Dräger, Lübeck, Germany) to maintain an end-tidal CO_2 concentration of ~ 5% (Capnosat; Dräger). Respiration was performed with 60% N_2O and 40% O_2. The right carotid artery and jugular vein were isolated and cannulated with eight French introducers (Rüsch, Kernen, Germany) to establish an extracorporeal circuit. For measurement of cardiovascular parameters, the following catheters were inserted and connected to the Ponemah acquisition and analysis system through Combitrans transducers (Combitrans; Braun, Melsungen, Germany) and Gould transducers (series 6600): a hollow catheter into the femoral artery for recording of arterial blood pressure; a venous catheter into the femoral vein for application of fluids and withdrawal of plasma samples; and two venous catheters into the lateral saphenous veins for drug application.

Cardiovascular parameters (e.g. arterial blood pressure, electrocardiogram [lead II, via a Gould ECG/Biotach transducer], heart rate, and rectal temperature) were monitored throughout the study with the Ponemah acquisition system; mean values of all parameters over an interval of 5 min were used for analysis.

Extracorporeal shunt

The setup of the extracorporeal shunt is shown in Fig. 1. The setting was modified from that of Makkar *et al.* [12]. Briefly, a tubular perfusion chamber (Plexiglas, manufactured in the Bayer Pharma AG laboratories according to Badimon *et al.* [13]) with an inner diameter of 2 mm was used for the perfusion experiments. Stainless steel stents of length 13 mm (Balloon-Expandable Stent System Ref. No. PR13250, BX Sonic; Cordis, Roden, the Netherlands) were expanded within the perfusion chamber to an outer diameter of 2 mm. Stents were mounted in the chamber, and covered with endothelium-denuded porcine aortic strips to obtain a watertight seal, and to mimic the contact area between stents and damaged vascular tissue. The arterial cannula was connected to the inlet of the perfusion chamber, and the outlet was connected to the venous catheter, with blood flow driven through the system by a variable-speed peristaltic pump (Masterflex L/S; Cole-Pharma Instruments, Chicago, IL, USA). A transit time Doppler flow probe (Ultrasonic Clamp On Transducer; Em-tec, Finning, Germany) was placed within the circuit after the pump to document continuous blood flow through the circuit. To maintain body temperature, the chamber and tubing were placed in a water bath at 37 °C.

Perfusion protocol

After the animals had been set up for hemodynamic recordings, a 30-min stabilization period was allowed to stabilize hemodynamics; the first stent was mounted in the tubular chamber (modified Badimon chamber [13]) and perfused with saline solution for 60 s at 37 °C. Subsequently, with a switch valve to prevent stasis, blood was circulated through the system; flow was regulated at 70 mL min^{-1} for 30 min, resulting in wall shear rates of 1485 s^{-1} at the chamber surface and 1748 s^{-1} at the stent surface (formula: shear [s^{-1}] = $4 \times \text{flow}/[\pi r^3]$; inner diameter 0.3 mm less than outer diameter).

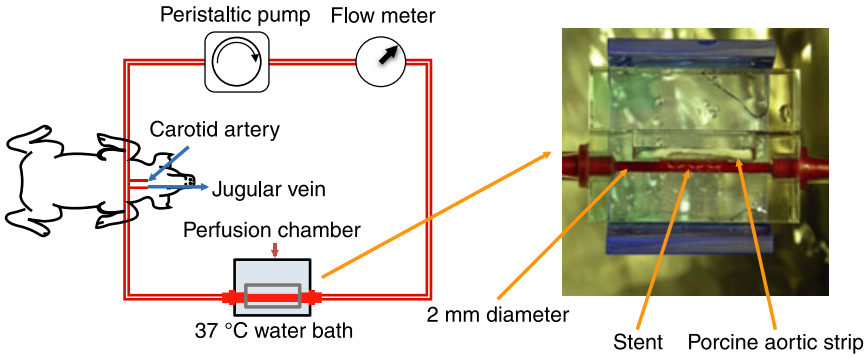


Fig. 1. Diagram of the extracorporeal perfusion system (adapted from Makkar *et al.* [12]). Expanded stents were mounted in the tubular chamber and exposed to flowing blood from the carotid artery. Blood flow was regulated by the peristaltic pump (70 mL min^{-1}) and returned to the jugular vein.

At the end of each 30-min perfusion period, 37 °C saline solution was circulated through the chamber for 60 s at 10 mL min^{-1} to wash blood from the stent and the perfusion system. At the completion of each perfusion period, stents were removed from the chamber. Digital images of stents and porcine aortic strips were obtained in lengthways and end-on profiles with a stereomicroscope and a digital camera.

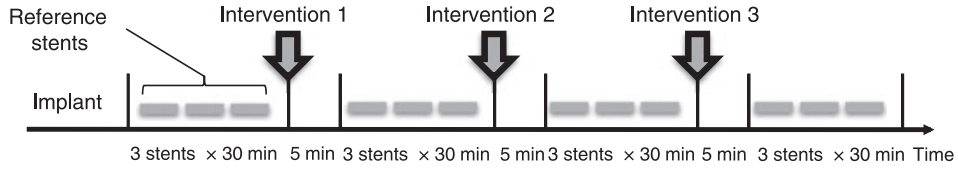
In-stent thrombus weight and aortic strip-adherent thrombus weight were determined. Total thrombus weights were calculated as the sum of in-stent thrombus weight and the thrombus weight adhering to the porcine aortic strips. The in-stent thrombus weight was determined as the total weight after perfusion minus the bare thrombus weight before exposure. The weight of thrombus adhering to the aortic strip was determined by scratching the adhered thrombus mass gently from the aortic strip and weighing the adhered thrombus mass only. Subsequently, the perfusion chamber and ex vivo circuit were cleaned with pipe cleaners, and perfused with saline solution for several minutes to wash off unattached cells and any visible blood before another stent was mounted. A total of 12 stents were perfused in each animal (Fig. 1). These were split into three initial reference stents followed by three groups of 'stent triples' under different treatment conditions. The initial stent triple (reference stents) in each animal was performed under naïve (baseline) conditions, and served as an internal stent thrombosis reference for each animal to control for any inter-animal differences. We validated our model by reconfirming the results of Makkar *et al.* [12] under our experimental conditions. As shown in Fig. 2, to evaluate the effect of rivaroxaban in our high-shear-induced stent thrombosis model, we performed a blinded, randomized animal study, in which animals were randomized to the five different groups. Different drugs were prepared by another technician, and handed over to the study technician in a blinded manner. Only after all relevant data had been analyzed for each animal were the animals allocated to their respective groups.

The animals (n = 34) were distributed across five groups (Fig. 2):

1 A vehicle control group (seven animals receiving all corresponding vehicle solutions used in the treatment groups, resulting in 21 stents per treatment);
2 A rivaroxaban dose–response group (eight animals receiving three increasing rivaroxaban doses [i.v. bolus followed by 0.11, 0.33 and 1.0 μg kg^{-1} min^{-1}] to mimic the steady-state plasma levels achieved under clinical conditions after oral treatment, resulting in 24 stents per treatment);
3 A rivaroxaban dose–response group in combination with 1.0 mg kg^{-1} ASA as single antiplatelet treatment (six animals, resulting in 18 stents per treatment);
4 A rivaroxaban dose–response group in combination with 1.0 mg kg^{-1} ASA and clopidogrel 0.5 mg kg^{-1} i.v. as DAT (seven animals, resulting in 21 stents per treatment);
5 A DAT group receiving ASA (1.0 mg kg^{-1}) and clopidogrel (0.5 mg kg^{-1}), which served as reference group to test the effect of the clinical standard-of-care in this model (six animals, resulting in 18 stents per treatment). Dosing for the DAT was chosen to match clinical human dosing as closely as possible, while allowing a suitable window to see any potential synergistic effects when it was used in combination with rivaroxaban.

Bioanalytics

Venous blood samples (1.2 mL of Li-Heparin for rivaroxaban and 1.2 mL of Li-Heparin with 3.6 g of sodium fluoride for ASA) were collected at 5 min before and 5, 10, 20, 30, 60 and



Group	Animal number	Reference	Intervention 1	Intervention 2	Intervention 3
Vehicle control	n = 7	Reference stents	Vehicle	Vehicle	Vehicle
Rivaroxaban	n = 8	Reference stents	0.11 μg kg^{-1} min^{-1}	0.33 μg kg^{-1} min^{-1}	1.0 μg kg^{-1} min^{-1}
Rivaroxaban + ASA	n = 6	Reference stents	0.11 μg kg^{-1} min^{-1} + 1.0 mg kg^{-1}	0.33 μg kg^{-1} min^{-1}	1.0 μg kg^{-1} min^{-1}
Rivaroxaban + ASA + clopidogrel	n = 7	Reference stents	0.11 μg kg^{-1} min^{-1} + 1.0 mg kg^{-1} + 0.5 mg kg^{-1}	0.33 μg kg^{-1} min^{-1}	1.0 μg kg^{-1} min^{-1}
Vehicle + ASA + clopidogrel	n = 6	Reference stents	Vehicle + 1.0 mg kg^{-1} + 0.5 mg kg^{-1}	Vehicle	Vehicle

Fig. 2. Perfusion protocol for the rivaroxaban study. ASA, acetylsalicylic acid.

90 min after the start of cumulative rivaroxaban infusions. Plasma concentrations of rivaroxaban in these samples were then determined. Plasma concentrations were also assessed at 5 min before and 90, 180 and 270 min after bolus injection of ASA. Bioanalytic analyses of rivaroxaban were performed at Bayer's bioanalytics department.

Plasma concentrations of rivaroxaban were determined by HPLC coupled with a tandem mass spectrometer (MS/MS, MDS Sciex API 2000; Applied Biosystems, Foster City, CA, USA), with a stable isotope-labeled internal standard.

Preparation and administration of drugs

Clopidogrel was extracted from Plavix 75-mg pills (Sanofi-Aventis, Frankfurt, Germany) and dissolved in a mixture of Transcutol (2-[2-ethoxyethoxy]ethanol; Gattefossé, Saint-Priest Cedex, France) and PEG400 (50 : 50). For i.v. boluses of ASA, Aspisol (Bayer Pharma AG, Leverkusen, Germany) dissolved in water was used. Both drugs were dissolved on the day of the experiment and administered as i.v. boluses at a volume of 50 μL kg^{-1}, followed by 1 mL of NaCl for ASA or 1 mL of Transcutol/PEG400/NaCl (25 : 25 : 50) to flush the catheter. Transcutol/PEG400 (50 : 50) at a volume of 0.15 mL kg^{-1} h^{-1}, accompanied by 0.25 mL kg^{-1} h^{-1} NaCl, was used as the rivaroxaban vehicle. Drug administration was performed in a blinded manner.

Bleeding time

Bleeding time and volume were determined before and after each stent triple. An ear vein puncture was performed with a 23G × 1.25-inch cannula. The time between incision and cessation of bleeding was recorded as bleeding time; blood was absorbed every 10 s with a swab. Bleeding volume was also assessed at each time point; the absorbed blood was eluted in a defined amount (4–48 mL, depending on the blood volume) of distilled water, and centrifuged (MinifugeRF; Heraeus, Osterode, Germany) at 2200 × ***g*** and 4 °C for 10 min. The supernatant was diluted with distilled water in 96-well plates, in line with the linear extinction coefficient range, and the hemoglobin concentration was photometrically measured at 420 nm with a double-monochromatic photometer (Tecan Safire, Durham, NC, USA). Results are shown as mean bleeding volume (μL) ± standard error of the mean (SEM).

Clotting time, thromboxane B_2 (TXB_2) determination, platelet aggregation, and blood cell count

At baseline and after each intervention (stent triple), venous blood samples were collected for determination of activated clotting time (ACT), prothrombin time (PT), activated partial thromboplastin time (APTT), thromboxane B_2 (TXB_2), platelet aggregation, and blood cell count. ACT, PT and APTT were determined with a Hemochron Jr Signature (Lamed, Munich, Germany).

TXB_2 was determined by mixing an arterial blood sample on a Thermomixer (Eppendorf, Hamburg, Germany) for 60 min at 37 °C. The serum was centrifuged twice at 8000 × ***g*** for 10 min, and TXB_2 was measured with a commercially available enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA), according to the manufacturer's instructions.

For measurement of aggregation, ex vivo blood samples were collected in 3.8% trisodium citrate solution after three, six, nine and 12 stents had been used. Platelet aggregation was measured by the impedance method in the Multiplate analyzer (Dynabyte Medical, München, Germany). A 300-μL volume of whole blood, including 4 mM Pefabloc (Pentapharm, Basel, Switzerland), was diluted 1 : 2 with 4 mM $CaCl_2$ in NaCl, and left to equilibrate for 3 min at 37 °C. Measurement was started by the addition of 60 μL (1 : 10 dilution) of 100 nM FXa (Kordia Life Sciences, Leiden, the Netherlands), 200 μM ADP, thromboplastin (Roche, Basel, Switzerland; diluted 1 : 8 and 1 : 4), 100 nM and 1 μM α-thrombin (Kordia Life Sciences), and 200 μg mL^{-1} and 250 μg mL^{-1} collagen reagent Horm (Nycomed, Linz, Austria).

Blood cell counts were performed with a Cell Dyn 3700 (Abbott Diagnostics, Wiesbaden, Germany).

Statistical analysis

Data are presented as mean ± SEM. The statistical difference between means was determined with an unpaired *t*-test vs. representative vehicle group. *P*-values of < 0.05, < 0.01 and < 0.001 were considered to indicate statistical significance.

Results

Stent thrombosis

Figure 1 shows the experimental setup of the technique used in the study. Figure 2 shows the protocol and schedule of the different treatments. It is of note that i.v. administration of the treatments allowed dose-escalating studies to be performed.

Representative pictures of the thrombi formed in our experimental system in response to the different treatments are shown in Fig. 3. Control stents were covered by a large and mostly occlusive thrombus. The thrombus showed the typical structure of a white (platelet) head and red (fibrin) tail. Administration of rivaroxaban resulted in a dose-dependent inhibition of thrombus formation. The rivaroxaban and ASA combination inhibited thrombus formation even more. Combined administration of rivaroxaban with ASA and clopidogrel almost completely abolished thrombus formation on the perfused stents. The quantitative comparison of the antithrombotic effects of the different treatments is shown in Fig. 4.

In our animal model, absolute thrombus weight in vehicle-treated stents showed a time dependency during the course of the experiment (Fig. 4). In the vehicle group, as compared with the mean thrombus weight across the three reference stents, in-stent thrombus weight decreased slightly during the interventions (from 10.9 ± 1.1 mg [reference stents] to 8.0 ± 1.1 mg

[intervention 1], 9.3 ± 1.4 mg [intervention 2], and 10.3 ± 1.6 mg [intervention 3]). To avoid different results with different vehicles in all groups independently of intervention, each of the different vehicles was administered in the vehicle group during each intervention. As compared with vehicle-treated animals, rivaroxaban significantly and dose-dependently reduced thrombus weight, starting at the lowest dose of 0.11 μg kg^{-1} min^{-1}. With rivaroxaban alone, thrombus weight was significantly reduced by 33% at the dose of 0.11 μg kg^{-1} min^{-1} ($P < 0.05$), by 47% at the dose of 0.33 μg kg^{-1} min^{-1} ($P < 0.01$), and by 66% at the highest dose of 1.0 μg kg^{-1} min^{-1} ($P < 0.001$). The addition of ASA to rivaroxaban produced greater inhibition of thrombus weight, which decreased by 48% at the dose of 0.11 μg kg^{-1} min^{-1} ($P < 0.01$), by 72% at the dose of 0.33 μg kg^{-1} min^{-1} ($P < 0.001$), and by 86% at the highest dose of 1.0 μg kg^{-1} min^{-1} ($P < 0.001$). Treatment with rivaroxaban in combination with ASA and clopidogrel was even more effective: thrombus weight was reduced by 59% at the dose of 0.11 μg kg^{-1} min^{-1} ($P < 0.01$), by 91% at the dose of 0.33 μg kg^{-1} min^{-1} ($P < 0.001$), and by 98% at the highest dose of 1.0 μg kg^{-1} min^{-1} ($P < 0.001$). Standard DAT consisting of 1.0 mg kg^{-1} ASA and 0.5 mg kg^{-1} clopidogrel produced a decrease in thrombus weight by a maximum of 79% ($P < 0.001$). As shown in Fig. 4, we also compared triple therapy consisting of rivaroxaban, ASA and clopidogrel with standard DAT. For the two higher doses of rivaroxaban (0.33 μg kg^{-1} min^{-1} and 1.0 μg kg^{-1} min^{-1}), triple therapy significantly decreased IST as compared with DAT alone. In addition, the combination of rivaroxaban and the antiplatelet ASA was similar in efficacy to DAT alone.

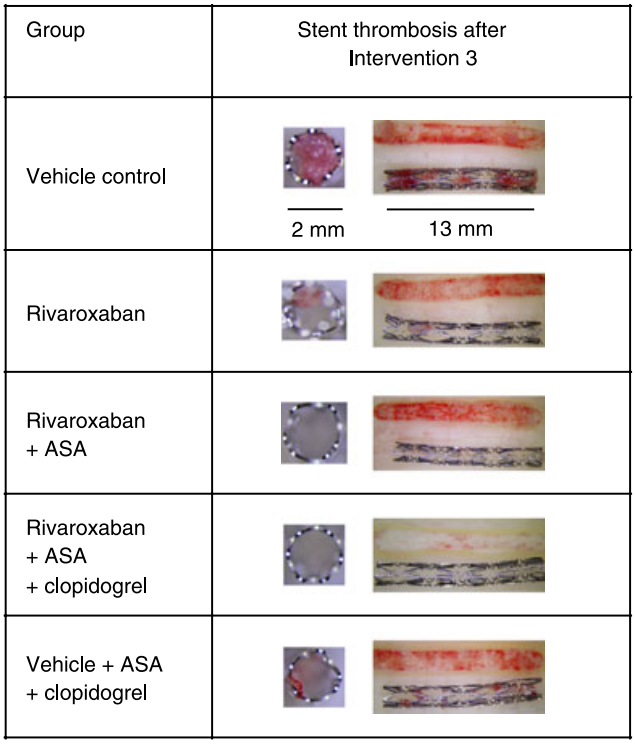


Fig. 3. Thrombus formation in the rivaroxaban study: lengthways view and end-on profile showing remaining in-stent thrombus in each treatment group at the maximal effect level. ASA, acetylsalicylic acid.

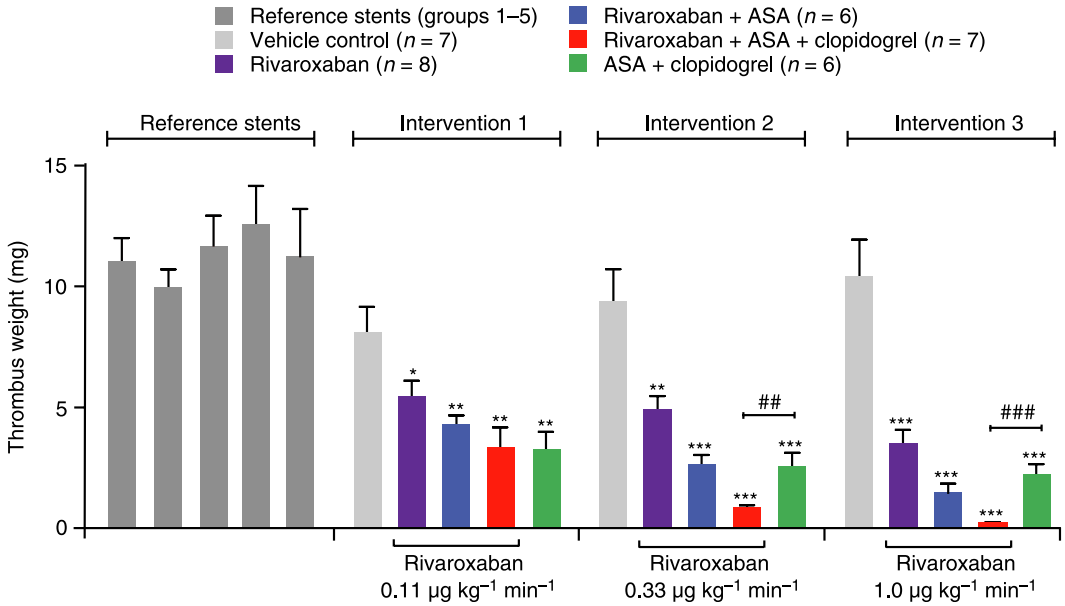


Fig. 4. Effects of rivaroxaban, alone and in combination with acetylsalicylic acid (ASA) and clopidogrel, on total thrombus weight (mg). Values shown are mean ± standard error of the mean. Each of the five bars in the reference stents (groups 1–5) corresponds to one group of animals used in the following intervention periods (group 1, vehicle control; group 2, rivaroxaban; group 3, rivaroxaban + ASA; group 4, rivaroxaban + ASA + clopidogrel; group 5, ASA + clopidogrel). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (unpaired *t*-test) vs. representative vehicle control group; $^{\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ (unpaired *t*-test) vs. representative ASA + clopidogrel group.

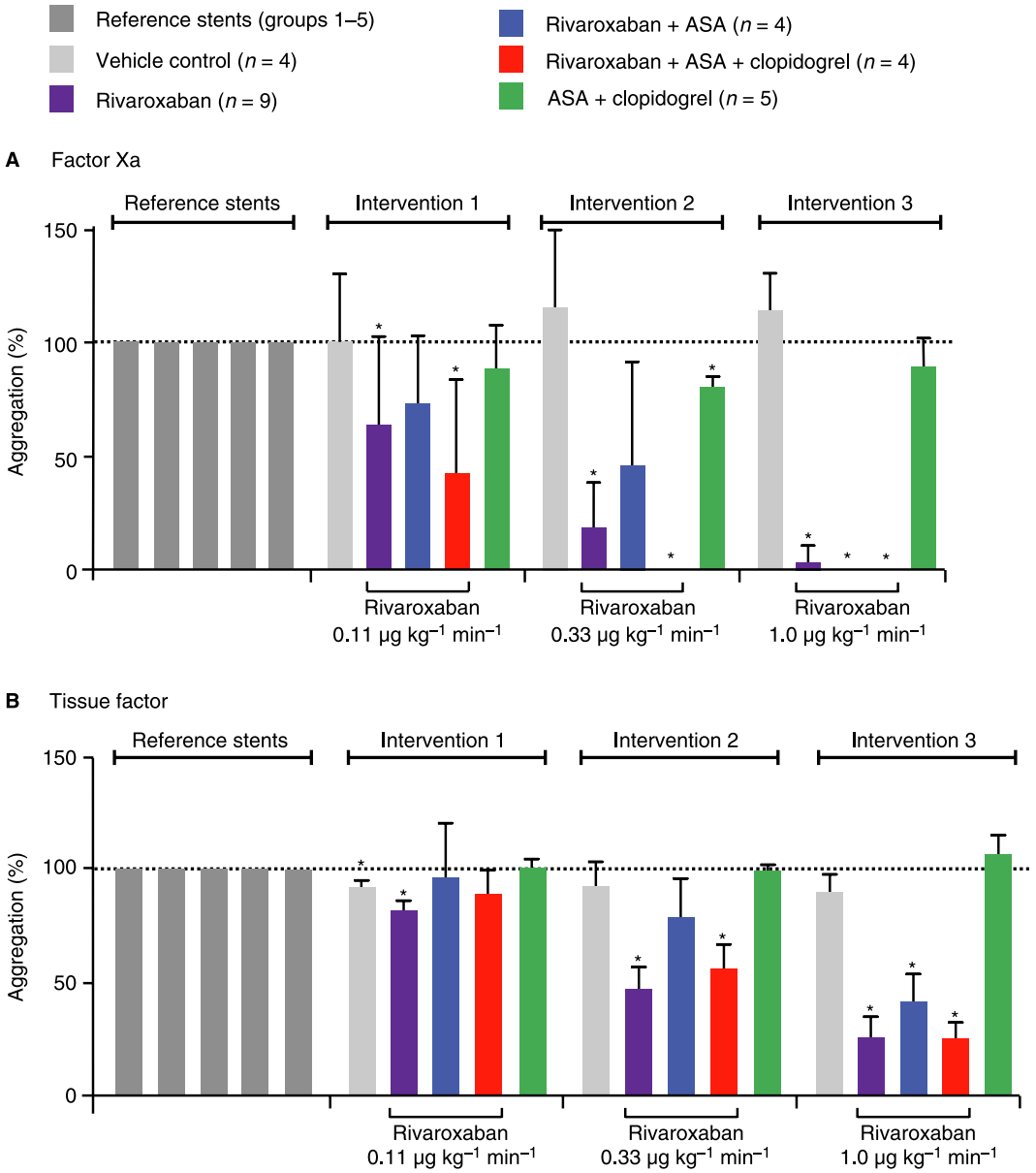


Fig. 5. Effects of different treatments on platelet aggregation. (A, B) Effects of rivaroxaban alone and in combination with acetylsalicylic acid (ASA) and clopidogrel on ex vivo factor Xa-induced and tissue factor-induced platelet aggregation. Values shown are mean ± standard error of the mean. (A) $*P < 0.05$ (unpaired *t*-test) vs. vehicle control; FXa 10 nM. (B) $*P < 0.05$ (unpaired *t*-test) vs. vehicle control; tissue factor 1 : 8. (C, D) Effects of rivaroxaban alone and in combination with ASA and clopidogrel on ex vivo ADP-induced and thrombin-induced platelet aggregation. Values shown are mean ± standard error of the mean. (C) $*P < 0.05$ (unpaired *t*-test) vs. vehicle control; ADP 20 µM. (D) $*P < 0.05$ (unpaired *t*-test) vs. vehicle control; thrombin 10 nM.

Platelet aggregation studies

The effects of the treatments on platelet reactivity were studied at different time points (Fig. 5A–D). Rivaroxaban induced a dose-dependent inhibition of FXa-induced, tissue factor-induced and thrombin-induced platelet aggregation. As expected, clopidogrel and ASA significantly inhibited ADP-induced platelet aggregation, indicating pharmacologically effective dosing. When added to clopidogrel and ASA, rivaroxaban appeared to further inhibit ADP-induced platelet aggregation. The addition of ASA to rivaroxaban did not cause any further significant inhibition of the aggregation induced by the used agonists as compared with rivaroxaban alone. In all ASA interventions, TXB_2 formation was maximally inhibited (between 91.3% and 98.6% inhibition vs. baseline values, data not shown), demonstrating effective dosing of ASA.

Hematologic studies

The quantitative effects of the study compounds on bleeding time are shown in Fig. 6. Only the highest rivaroxaban dose of 1.0 µg kg^{-1} min^{-1} in combination with 1.0 mg kg^{-1} ASA and 0.5 mg kg^{-1} clopidogrel induced a significant prolongation of

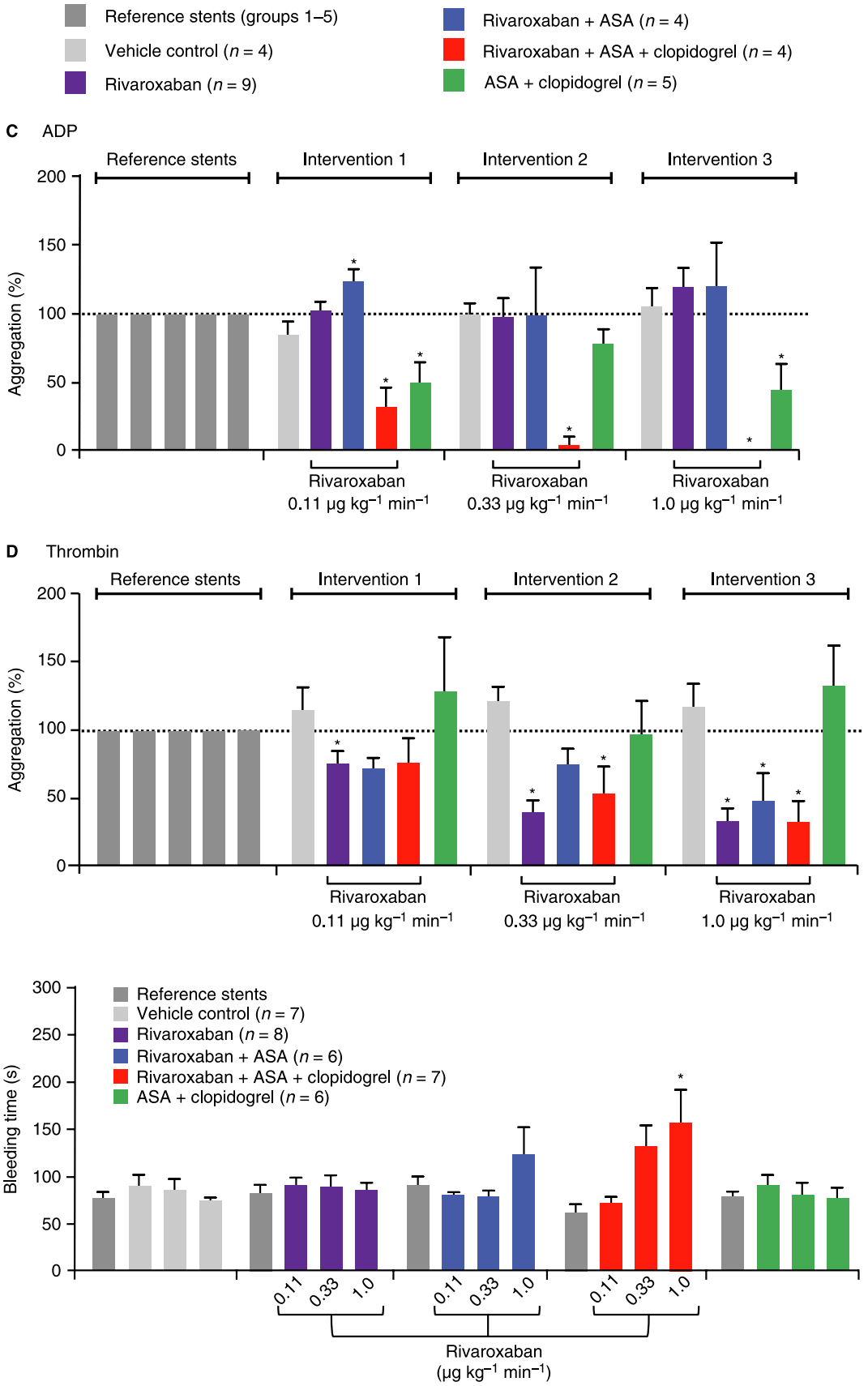


Fig. 6. Effects of study drugs on bleeding time measured by ear vein puncture. Values shown are mean ± standard error of the mean. *$P < 0.05$ (unpaired *t*-test) vs. representative vehicle control group. ASA, acetylsalicylic acid.

bleeding time ($P < 0.05$). The combination of rivaroxaban and ASA did not significantly increase bleeding time. No significant effect on bleeding volume by ear vein puncture was observed in any study set. Apart from the invasiveness of the procedures, we did not see any bleeding complications (e.g. blood loss and blood pressure drop).

The effects on PT are shown in Fig. 7A. Treatment with vehicle, ASA and clopidogrel showed no effects on PT. In contrast, rivaroxaban dose-dependently prolonged PT. The addition of ASA, as well as ASA plus clopidogrel, did not affect this PT prolongation. Similar effects on ACT were observed (Fig. 7B). In addition, higher rivaroxaban doses (0.33 μg kg^{-1} min^{-1} and 1.0 μg kg^{-1} min^{-1}) could be monitored by a prolongation of APTT (data not shown). No significant effects on blood cell count, blood pressure or heart rate were observed in any of the study sets (data not shown), and similar plasma levels of rivaroxaban were seen in all groups (Fig. 8).

Discussion

This study was performed to evaluate the effect of the direct FXa inhibitor rivaroxaban, an orally administered anticoagulant, alone and in combination with the antiplatelet compounds ASA and clopidogrel, on the inhibition of high-shear-mediated stent thrombosis. Several features differentiate new agents such as rivaroxaban from warfarin, which is considered to be the gold standard in oral anticoagulation. Among the advantages offered by these new anticoagulants as compared with warfarin are: predictability of fixed doses; no requirement for routine

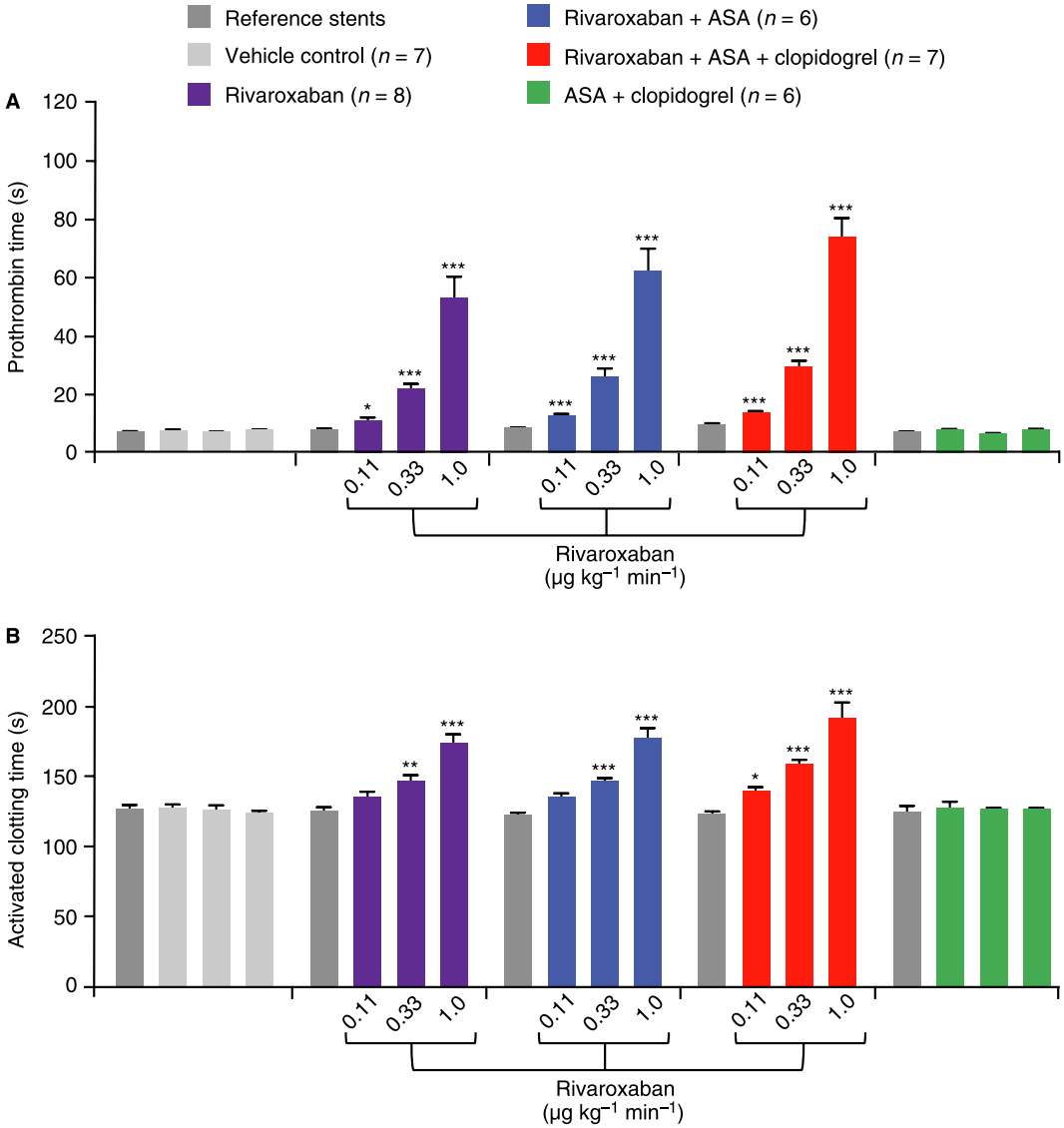


Fig. 7. Effects of study drugs on prothrombin time (A) and on activated clotting time (B). Values shown are mean ± standard error of the mean. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (unpaired *t*-test) vs. representative vehicle control group. ASA, acetylsalicylic acid.

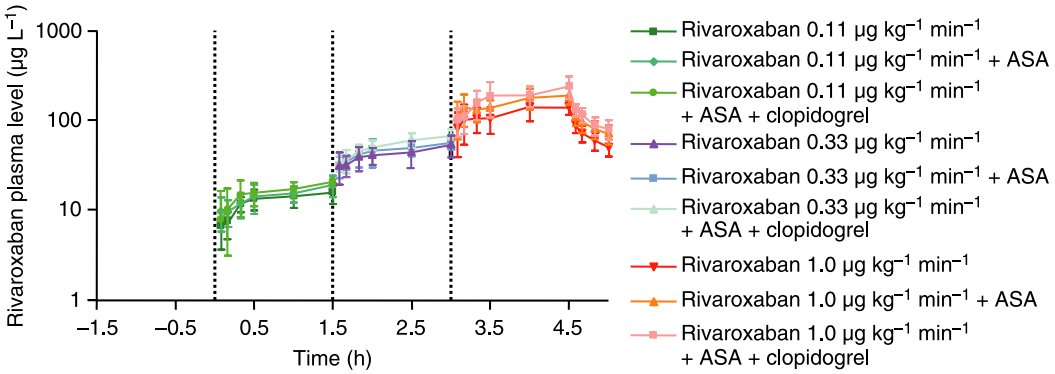


Fig. 8. Plasma levels of rivaroxaban measured following infusion of the drug in different groups. Values shown are mean ± standard error of the mean. ASA, acetylsalicylic acid.

coagulation monitoring; and few food and drug interactions. Our principal finding was that rivaroxaban, as an anticoagulant, shows dose-dependent efficacy for the inhibition of early stent thrombosis. Under our experimental conditions, when used in combination with either ASA or ASA and clopidogrel, rivaroxaban reduced stent thrombosis more effectively than ASA and clopidogrel without rivaroxaban. Results from the macroscopic analysis and the gravimetric determination of in-stent thrombus weight demonstrated that the triple therapy of rivaroxaban, ASA and clopidogrel almost completely abolished thrombus formation, with even the contact area between stents and the endothelium-denuded aortic strips being devoid of any thrombus.

In the study, the pharmacodynamic effect of rivaroxaban affected various clotting assays in porcine plasma as well as platelet aggregation in whole blood. In ex vivo aggregation assays, plasma samples from rivaroxaban-treated animals showed dose-dependent inhibition of FXa and tissue factor, as well as thrombin-induced platelet aggregation. ADP-induced platelet aggregation was inhibited by clopidogrel, an effect that seems to have been enhanced by the presence of rivaroxaban.

Plasma levels of rivaroxaban were monitored in all experimental groups, and correlated closely with increases in PT, ACT, and, with the highest rivaroxaban doses, APTT (data not shown). Steady-state plasma levels for the three cumulative doses of rivaroxaban were similar to the clinically relevant peak–trough plasma concentrations in patients who receive a daily dose of 5–20 mg of oral rivaroxaban [14]. Therefore, one could expect the beneficial effects of rivaroxaban seen in this model to be translated to the clinical setting, as already suggested in the ATLAS ACS 2 TIMI 51 trial.

The model in this study utilized a modified Badimon chamber [13]; this has been used extensively to study the mechanisms of experimental thrombosis and as a preclinical model to study the effects of DAT. This is the first study to demonstrate the additive effects of an anticoagulant in addition to DAT in this model. This stent thrombosis model provided reproducible and stable results, even after multiple perfusions over time. Although the relevance of this acute model with respect to clinical outcome remains to be defined, the effects of platelet inhibitors and anticoagulants, as well as their combinations, could be assessed under these experimental conditions. The efficacy of the current standard of care (ASA and clopidogrel) was demonstrated in this model. Additionally, we were able to demonstrate not only the efficacy of rivaroxaban as an anticoagulant alone, but also the enhanced efficacy of rivaroxaban in combination with either a single antiplatelet (ASA) or both ASA and clopidogrel. Within this dual pathway context, rivaroxaban was highly efficacious in preventing stent thrombosis.

As expected, triple therapy caused an increase in bleeding time, particularly with the highest rivaroxaban dose. Nevertheless, episodes of obvious bleeding at the surgical sites (even in light of the invasiveness of the procedure) were not observed. Therefore, the relevance of the observed increases in bleeding time and in hematologic parameters (PT and ACT) within the context of highly enhanced efficacy for prevention of stent thrombosis remains to be fully evaluated. However, clinical trials of triple antithrombotic therapy in patients with ACS have been performed, including the ATLAS ACS 2 TIMI 51 trial with rivaroxaban [10]. Here, it was shown that rivaroxaban reduced the risk of the composite endpoint of death from cardiovascular causes, myocardial infarction, or stroke. The risk of major bleeding and intracranial hemorrhage was increased, but the risk of fatal bleeding or fatal intracranial hemorrhage was not, even under triple therapy consisting of rivaroxaban, low-dose ASA, and thienopyridine (either clopidogrel or ticlopidine). Although it was not part of the formal statistical hierarchy, the ATLAS ACS 2 TIMI 51 study also reported a significantly reduced stent thrombosis rates with rivaroxaban vs. placebo, supporting the possible role of safe, orally available anticoagulants in this clinical complication.

Platelet aggregation is a crucial aspect of the formation of occlusive stent thrombosis. However, in-stent thrombi are both platelet-rich and fibrin-rich, underlining the role of the coagulation cascade in stabilizing clots [6–8]. There is therefore an underlying mechanistic explanation for the additive effect of rivaroxaban in combination with single antiplatelet therapy or

DAT for the prevention of stent thrombosis, as observed in our study.

Our study therefore established the efficacy of rivaroxaban, a potent, oral, direct FXa inhibitor, for the inhibition of stent thrombosis under high-shear conditions within our experimental model using anesthetized minipigs. High-risk clinical situations such as small-vessel stenting and incorrectly placed stents are characterized by high shear stress, and are important sources of early stent thrombosis, which occurs at a rate of at least 1.4% [1–3,15].

Taking into consideration that stent thrombosis also occurs in drug-eluting stents at later time points [4,6], an orally available, effective anticoagulant therapy for patients who have undergone stent placement that could be administered for longer than 12 months (as is recommended for DAT) might further improve patient outcomes. Our study design consisted of an ex vivo extracorporeal circuit in which stent thrombosis occurred within 30 min. By contrast, the peak incidence of stent thrombosis in patients is seen within the first 3–4 days [3]. However, acute stent thrombosis is occasionally observed, and the role of acute platelet deposition in subacute and late stent thrombosis may be underestimated. Our acute model excludes the chronic effects of the drugs on vessel walls, which might affect in-stent thrombus formation. Nevertheless, to simulate the underlying vascular damage, we used endothelium-denuded porcine aortic strips, in close contact with the bare metal stent, to resemble the clinical situation as closely as possible.

Other limitations of this study include the i.v. administration of study drugs; in the clinical setting, these drugs would be administered orally. Therefore, differences in bioavailability and time to maximum plasma concentration would need to be considered in making this transition. Additionally, denuded aortic strips are non-coronary arteries, and were used in this study for practical reasons.

Nevertheless, the results of our study demonstrate the effect of rivaroxaban in inhibiting stent thrombosis under high-shear conditions. Combined therapy composed of rivaroxaban and ASA, or the triple therapy approach of rivaroxaban, ASA, and clopidogrel, may be a useful antithrombotic strategy in high-risk clinical situations such as small-vessel stenting, inadequate stent placement, and the presence of a thrombus. Moreover, an oral, direct FXa inhibitor such as rivaroxaban can also reduce platelet aggregation, as shown by the effect on tissue factor-induced platelet aggregation. Thus, direct FXa inhibitors can be seen as (indirect) antiplatelet compounds, owing to the inhibition of thrombin generation. Our study also indicates that a combination of ASA and a FXa inhibitor has similar effectiveness as the dual antiplatelet combination of ASA and clopidogrel for the inhibition of IST. The additional administration of a selective FXa inhibitor offers the dual benefit of inhibiting both platelet aggregation and the coagulation cascade, and should be more effective than DAT alone for the prevention of IST.

In conclusion, our data demonstrate the effectiveness of rivaroxaban in the prevention of arterial thrombosis, and are in line with the results of the ATLAS ACS 2 TIMI 51 trial, showing its clinical benefits in secondary ACS prevention. Taken together, these data support further testing of rivaroxaban in patients with ACS after percutaneous coronary intervention.

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Disclosure of conflict of interests

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