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Dabigatran but not rivaroxaban or apixaban treatment decreases fibrinolytic resistance in patients with atrial fibrillation

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Abstract

Introduction. Most anticoagulants stimulate fibrinolysis in vitro through mechanisms dependent on and independent of thrombin activatable fibrinolysis inhibitor (TAFI). We evaluated the effect of dabigatran, rivaroxaban and apixaban treatment on plasma fibrinolysis in patients with non-valvular atrial fibrillation.
Methods and Results. Patients treated with dabigatran etexilate (n = 22), rivaroxaban (n = 24) or apixaban (n = 22) were studied. Plasma was obtained before (trough) and 2 hours after drug intake (peak). Fibrinolytic resistance of clots exposed to exogenous tissue plasminogen activator was significantly lower in peak than in trough samples and correlated with drug concentration only in dabigatran group. Moreover, fibrinolytic resistance at peak was lower in dabigatran than in rivaroxaban and apixaban groups. This difference disappeared if the TAFI pathway was inhibited. Thrombin generation and TAFI activation were markedly lower in peak than in trough samples in all three groups. However, TAFIa levels in trough and peak samples were significantly lower in dabigatran group than in rivaroxaban and apixaban groups. Circulating levels of prothrombin fragment F1+2 (reflecting in vivo thrombin generation) and plasmin-antiplasmin complex (reflecting plasmin generation) were not or barely influenced by drug levels in all groups.
Conclusions. Our data suggest that dabigatran, contrary to rivaroxaban and apixaban, reduces fibrinolytic resistance by virtue of its greater impact on TAFI activation. The profibrinolytic effect of dabigatran may play a role locally, at sites of fibrin formation, by making the nascent thrombus more susceptible to plasminogen-dependent degradation.

Keywords: anticoagulants, coagulation, fibrinolysis, thrombin, thrombin activatable fibrinolysis inhibitor

Introduction

Dabigatran, rivaroxaban and apixaban are target-specific direct oral anticoagulants (DOACs). Dabigatran is a reversible inhibitor of thrombin, which is formed in vivo after intake of the prodrug, dabigatran etexilate [1]. Rivaroxaban and apixaban are highly specific reversible inhibitors of factor Xa [2,3]. Several large clinical trials have documented the efficacy and safety of DOACs in diverse thromboembolic diseases [4], and the drugs are now licensed for the prevention of venous thromboembolism after knee and hip replacement, the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation and for the treatment of acute venous thromboembolism.

In vitro, most anticoagulants, including dabigatran [5] and rivaroxaban [6], have been shown to decrease fibrinolytic resistance of plasma and/or blood clots through the blockade of the antifibrinolytic effects brought about by thrombin, suggesting that the hastening of fibrin removal might contribute to the antithrombotic activity of the drugs [7]. Thrombin, besides its role in platelet activation and fibrin formation, is a key player in the protection of the haemostatic plug against premature lysis. In fact, thrombin is able to delay or even halt the fibrinolytic process through multiple mechanisms, which include: 1) a direct effect on fibrin structure, whereby the higher the thrombin concentration the more tightly packed and lysis-resistant the clot [8]; 2) the activation of factor XIII, which is essential for fibrin cross-linking and for the covalent binding of α2-antiplasmin to fibrin, which is one of the most powerful anti-fibrinolytic mechanisms [9,10]; 3) the activation of thrombin activatable fibrinolysis inhibitor (TAFI), a plasma procarboxypeptidase (also known as plasma procarboxypeptidase U or B) that, once activated (TAFIa), removes the C-terminal lysines from partially degraded fibrin, thereby reducing the binding of tissue-type plasminogen activator (t-PA) and plasminogen to the clot and, therefore, plasmin formation [11]. Whether DOACs decrease fibrinolytic resistance in clinical setting is unknown and hard to anticipate because patients under anticoagulation are generally old and frequently affected by several diseases, and thus may present with a compromised fibrinolytic system [12,13]. Moreover, the expression of thrombomodulin (TM) by vascular endothelial cells might interfere with the activity of direct thrombin inhibitors, as suggested by in vitro studies. It has indeed been reported that dabigatran and other thrombin inhibitors make the clots more resistant to fibrinolysis if TM is present in the clot lysis model [5,14,15]. Moreover, direct thrombin inhibitors have been shown to enhance thrombin generation in plasma containing TM [16,17] and in some animal models [17,18],raising concerns about a possible prothrombotic risk associated with dabigatran treatment. We performed a study in patients with non-valvular atrial fibrillation treated with dabigatran, rivaroxaban or apixaban to investigate 1) the effect of DOACs on fibrinolytic resistance; 2) the possible influence of TM; 3) the in vivo changes of fibrinolysis markers.

Materials and Methods

Patients and sample collection

Sixty-eight patients with non-valvular atrial fibrillation (NVAF) were enrolled at the Haemostasis and Thrombosis Center of Azienda Ospedaliera "Istituti Ospitalieri", Cremona, Italy. Patients received dabigatran etexilate (Pradaxa®, n = 22), rivaroxaban (Xarelto®, n = 24), or apixaban (Eliquis®, n = 22) according to AIFA (Agenzia Italiana del Farmaco) prescription criteria for the prevention of stroke and systemic embolism in NVAF. Venous blood was collected into 0.109 M citrate using Becton Dickinson coagulation tubes (BD, USA). Two samples were collected in the same morning, one just before drug intake (trough sample) and one 2 h after drug intake (peak sample). Blood was immediately centrifuged at 1200 g for 15 min at room temperature, and the resulting plasma was collected, snap frozen and stored at -80°C until analysis.
Dabigatran concentration in patients' plasma was measured by diluted thrombin time (STA-thrombin, Diagnostica Stago, Asnieres, France) on a magneto-mechanical coagulation analyzer (STA-R, Diagnostica Stago) as described [19]. Rivaroxaban and apixaban concentrations were measured by a chromogenic anti-Xa assay (STA®-Liquid Anti-Xa, Diagnostica Stago). The study conformed to the Declaration of Helsinki and informed written consent was obtained from each patient. The study protocol was approved by the institutional Ethic Committee (42-2014-OSS_FARM-CR27).

Proteins and reagents

Single-chain recombinant t-PA was from Boehringer Ingelheim (Florence, Italy); human thromboplastin (Recombiplastin) was from Instrumentation Laboratory (Milan, Italy); bovine fibrinogen and potato tuber carboxypeptidase inhibitor (PTCI) were from Sigma (Milan, Italy). Rabbit thrombomodulin (TM) and reptilase ST were from American Diagnostica (Pfungstadt, Germany).

Plasma clot lysis assay

The lysis of tissue factor-induced plasma clots exposed to exogenous t-PA was studied with a turbidimetric assay as described [5], with minor modifications. One hundred µL plasma, 10 µL thromboplastin (1:1000, final dilution, corresponding to approximately 6 pM tissue factor), 10 µL t-PA (30 ng/mL, final concentration, f.c.), and 20 µL Tris-NaCl buffer, were added to microplate wells, after which the clotting reaction was started with 100 µL $CaCl_2$ (8.3 mM, f.c.). The plate was incubated at 37°C, and the changes in optical density at 405 nm were measured every minute in a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA). Clot lysis time was defined as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. Where indicated, experiments were performed

in the presence of the specific TAFIa inhibitor PTCI (25 µg/mL, f.c.), or upon addition of the TAFI activation cofactor TM (4 nM, f.c.). In some experiments, clotting was induced by the thrombin-like enzyme reptilase [5] to avoid thrombin generation and TAFI activation.

TAFIa generation

Thrombin-mediated TAFIa generation was assessed in plasma by a two-stage functional assay as previously described [20], with minor modifications. Plasma was defibrinated by reptilase (1:50) for 1 hour at 37°C. Then, a mixture similar to that used for clot lysis assay, except for the absence of t-PA, was prepared in a test tube and incubated at 37°C. After 10 min, unless otherwise specified, an aliquot was withdrawn, mixed with hirudin (200 U/mL, f.c.; Abbott GmbH, Ludwigshafen, Germany,) to stop TAFI activation, and kept on melting ice until tested. TAFIa activity was evaluated as the ability to prolong the lysis time of purified fibrin clots. Thirty-five µL of sample were added to a microplate well along with 25 µL bovine fibrinogen (830 µg/mL, f.c.), 10 µL t-PA (30 ng/mL, f.c.) and 40 µL Tris-buffer, after which clot formation was induced by 10 µL reptilase (1:50, final dilution). The plate was read every minute at 405 nm at room temperature (to reduce the temperature-dependent TAFIa decay) and lysis times were calculated as described above. PTCI served as a reference for the absence of TAFI activity and results were expressed as prolongation of lysis time over the PTCI-containing sample. Experiments on pooled normal plasma containing increasing amounts of TAFIa showed a linear correlation between PTCI-sensitive lysis time prolongation and TAFIa concentration ($r = 0.994$).

Thrombin generation

Thrombin generation was evaluated by the calibrated automated thrombinography (CAT) method developed by Hemker et al [21]. Where indicated, experiments were performed in the presence of 4 nM TM. Because dabigatran inhibited thrombin bound to α2-macroglobulin [22], neither the sample-specific calibrator (test plasma spiked with α2-macroglobulin-thrombin) nor the Thrombinoscope software could be used in dabigatran-containing plasma samples. Therefore, we manually calculated the thrombin generation curve based on the velocity of fluorescence increase (first derivative), and measured the time to thrombin increase (lag time), peak signal and area under the curve (endogenous thrombin potential, ETP). Due to the lack of calibrators, the latter two parameters were expressed in arbitrary units (AU). Manual calculations could not be used for rivaroxaban and apixaban samples because of the bias caused by the cleavage of the fluorogenic substrate by thrombin-α2-macroglobulin complex. Therefore, CAT parameters in these samples were calculated by the Thrombinoscope software.

ELISA assays

The following biomarkers were measured in patients' plasma by commercially available ELISAs according to the manufacturers' instructions: activated TAFI (Asserachrom TAFIa/ai, Diagnostica Stago); prothrombin F1+2 (Enzygnost F1+2, micro, Siemens Healthcare Diagnostics Products, Marburg, Germany); and plasmin/α2-antiplasmin complex (Technozyme PAP, Technoclone GmbH, Vienna, Austria).

Statistical analysis

Data are presented as mean ± SD or median and interquartile range depending on distribution, as assessed by D'Agostino-Pearson test. Differences between trough and peak samples were analyzed by the Wilcoxon test for paired samples. Differences among groups by Kruskal-Wallis test followed by pairwise comparison according to Conover or by one-way ANOVA and Student-Newman-Keuls as post-hoc test. Correlations between variables were assessed by the Spearman rank correlation test. Statistical analyses were carried out using the MedCalc software (version 14.8.1). Concerning the study power, we calculated that 20 patients per group were needed to detect a 15% paired difference in lysis time between peak and trough samples ($\beta = 0.8$; $\alpha = 0.05$), assuming a standard deviation of the difference equal to 150% of the difference.

Results

Patients' characteristics

The main characteristics of patients are summarized in Table 1. The three groups were matched for age and gender, BMI, $CHADS_2$ score, and comorbidities. Only previous acute myocardial infarction was less frequent in dabigatran group, though not statistically significant. Drug concentrations were markedly higher at peak than at trough, the difference in median values ranging from 2 (apixaban) to 7-fold (rivaroxaban).

Table 1. Main characteristics of patients

	Dabigatran n = 22	Rivaroxaban n = 24	Apixaban n = 22	P
Age (yr)	73.4 ± 7.5	75.7 ± 7.4	76.1 ± 6.5	0.43
Weight (Kg)	75.5 ± 14.0	71.0 ± 20.5	75.5 ± 18.2	0.12
BMI	25.8 ± 6.8	25.2 ± 5.3	27.9 ± 5.5	0.28
Gender (M/F)	14/8	13/11	10/12	0.48
$CHADS_2$ score	1.64 ± 1.09	2.1 ± 1.26	2.2 ± 1.31	0.24
Daily dose of drug (n)	2*110 mg (12) 2*150 mg (10)	15 mg (3) 20 mg (21)	2*2.5 mg (4) 2*5 mg (18)	NA
Previous stroke or TIA (%)	18.2	16.6	18.2	0.98
Previous AMI (%)	4.5	29.2	22.7	0.09
Diabetes mellitus (%)	9.1	12.5	18.2	0.66
Hypertension (%)	68.2	70.8	81.8	0.55
Drug concentration at trough (ng/mL)	60.5 [39-130]	30.0 [20.5-50.5]	119 [67-161]	NA
Drug concentration at peak (ng/mL)	152 [92-308]*	212 [148-303]*	234 [168-292]*	NA

Differences among groups were tested by one-way ANOVA or Kruskal-Wallis test, as appropriate. NA, not applicable;
*, $P \leq 0.0001$ versus trough by Wilcoxon test for paired samples.

Fibrinolytic resistance of plasma clots

Fibrinolytic resistance was significantly lower (shorter lysis time) in peak than in trough samples in dabigatran but not in rivaroxaban and apixaban groups (Fig. 1A). Moreover, clot lysis time was significantly and inversely correlated only with the plasma levels of dabigatran, indicating that fibrinolytic resistance decreased as the dabigatran concentration increased (Table 2). Intergroup comparison revealed that clot lysis time at peak in dabigatran group was significantly shorter than the corresponding values in rivaroxaban and apixaban groups (Fig. 1A).

Table 2. Correlations between biomarkers and drug concentration

	Dabigatran		Rivaroxaban		Apixaban	
	rho	P	rho	P	rho	P
Ex vivo assays						
Lysis time	-0.409	0.005	-0.107	0.47	0.192	0.21
TM lysis ratio	0.852	< 0.0001	-0.190	0.23	-0.187	0.25
CAT parameters						
ETP	-0.700	< 0.0001	-0.613	< 0.0001	-0.386	0.010
Peak thrombin	-0.542	0.0001	-0.88	< 0.0001	-0.654	< 0.0001
Lag time	0.334	0.029	0.815	< 0.0001	0.686	< 0.0001
TAFI activation	-0.471	0.001	-0.623	< 0.0001	-0.380	0.011
In vivo markers						
F1+2	0.134	0.38	-0.148	0.32	-0.310	0.041
PAP	0.281	0.065	-0.119	0.89	0.271	0.075
TAFIa/ai	0.131	0.39	-0.098	0.51	-0.134	0.31

TM lysis ratio, lysis time prolongation induced by thrombomodulin; CAT, calibrated automated thrombinography; ETP, endogenous thrombin potential; TAFI activation, TAFIa activity detected 10 minutes after clotting activation by 6 pM tissue factor; F1+2, prothrombin fragment 1+2; PAP, plasmin-antiplasmin complex; TAFIa/ai, activated TAFI (TAFIa) plus its inactive derivative TAFIai. Correlations were assessed by the Spearman rank correlation test.

In dabigatran group, the difference in lysis time between peak and trough samples became much smaller upon addition of the TAFIa inhibitor PTCI, even though it remained statistically significant due to the paired comparison (Fig. 1B). Under this condition, a small difference in clot lysis time was also observed in rivaroxaban group.

To quantify the contribution of TAFI to fibrinolysis, we calculated the PTCI ratio (PTCI-R), which is given by the ratio between lysis times in the absence and in the presence of PTCI. In dabigatran group, PTCI-R was significantly lower in peak than in trough samples, indicating a lesser activation of TAFI in the former (Fig. 1C). On the contrary, PTCI-R at peak was similar or slightly higher than PTCI-R at trough in apixaban and rivaroxaban groups, respectively. PTCI-R values at peak and trough were significantly lower in dabigatran as compared to either rivaroxaban or apixaban group.

In all groups, no difference in fibrinolytic resistance between peak and trough samples was observed when clots were generated by the thrombin-like enzyme reptilase (not shown).

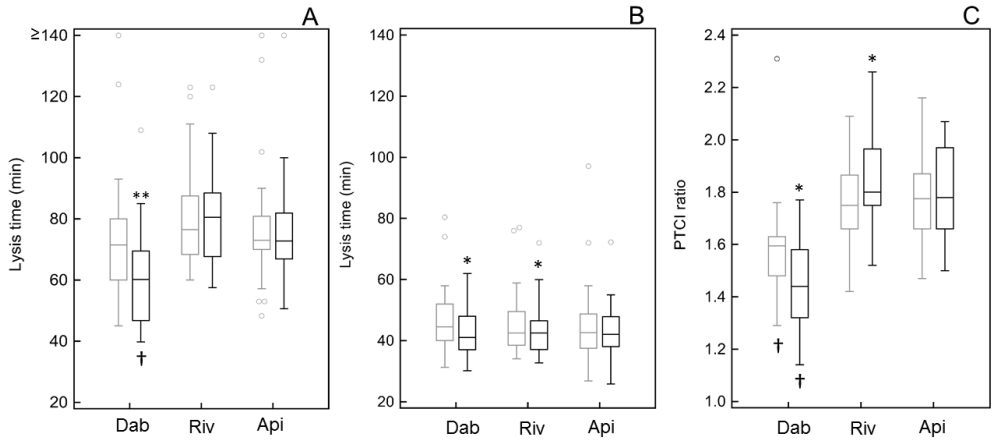

Fig. 1. Fibrinolytic resistance of plasma clots of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). Fibrinolytic resistance (expressed as lysis time) of TF-induced plasma clots to exogenous t-PA was assessed by a turbidimetric assay in samples taken at trough (grey boxes) and peak drug concentrations (black boxes). A, global fibrinolytic resistance; B, fibrinolytic resistance in the presence of the TAFIa inhibitor PTCI; C, contribution of TAFI to fibrinolysis calculated as PTCI ratio (ratio of lysis times in the absence and in the presence of PTCI). **, P = 0.0001 and *, P < 0.005 versus trough (Wilcoxon test); †, P < 0.05 versus corresponding samples of rivaroxaban and apixaban groups (Kruskal-Wallis test followed by pairwise comparison according to Conover).

Thrombin and TAFIa generation

ETP and maximum thrombin activity were reduced and lagtime prolonged in peak as compared to trough samples in all groups (Fig. 2). The most marked differences in thrombin generation were observed in rivaroxaban group. All CAT parameters were significantly correlated with drug concentration in all groups (Table 2). Intergroup comparison could not be carried out because of the different method used to calculate CAT parameters in dabigatran group.

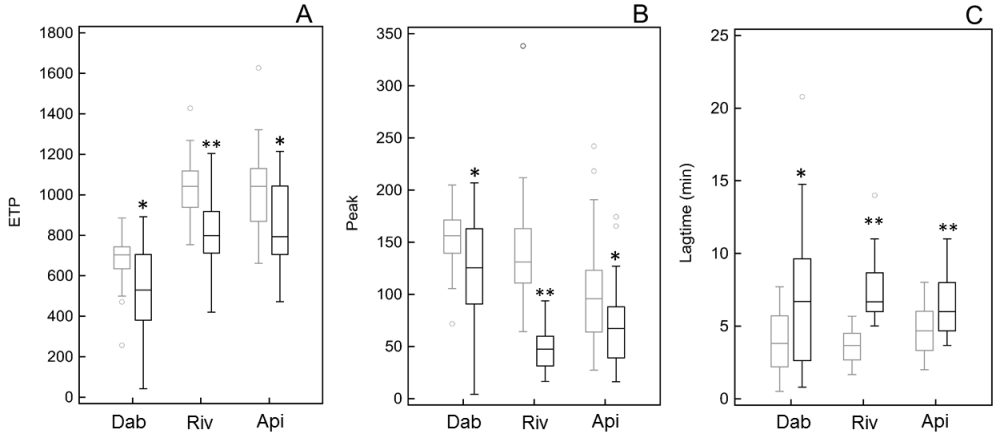

Fig. 2. Thrombin generation in plasma of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). Thrombin generation was evaluated by the calibrated automated thrombinography (CAT) in samples taken at trough (grey boxes) and peak drug concentrations (black boxes). Endogenous thrombin potential (ETP), maximum thrombin concentration (Peak) and Lag time are illustrated in panels A, B, and C, respectively. For dabigatran samples, ETP and peak are expressed in arbitrary units because of the unavailability of sample-specific calibrators (see methods for details). For rivaroxaban and apixaban groups, ETP and peak are expressed as nM*min and nM, respectively. **, P ≤ 0.0001 and *, P ≤ 0.001 versus trough (Wilcoxon test).

The extent of TAFI activation under conditions similar to clot lysis assay (except for t-PA addition) was evaluated by a fibrinolysis-based assay. The amount of TAFIa generated at 10 min after clotting activation was lower in peak than in trough samples in all groups (Fig. 3A) and significantly correlated with drug concentration (Table 2). However, in dabigatran group, TAFIa levels were significantly lower than in rivaroxaban and apixaban groups at both peak and trough drug concentrations. In some samples we also evaluated TAFIa generation over time to better appreciate the differences among groups. At trough (Fig. 3B), the highest concentration of TAFIa was reached at 10 min in all groups, but the levels of TAFIa measured in dabigatran samples were dramatically lower than those found in rivaroxaban and apixaban samples at all time points. The same was true for peak samples (Fig. 3C), except that the time to reach the maximum concentration of TAFIa was delayed in anti-Xa groups, meaning that the difference in TAFIa generation between the latter and dabigatran group was greater than that illustrated in Fig. 3A, which shows the TAFIa levels at 10 min.

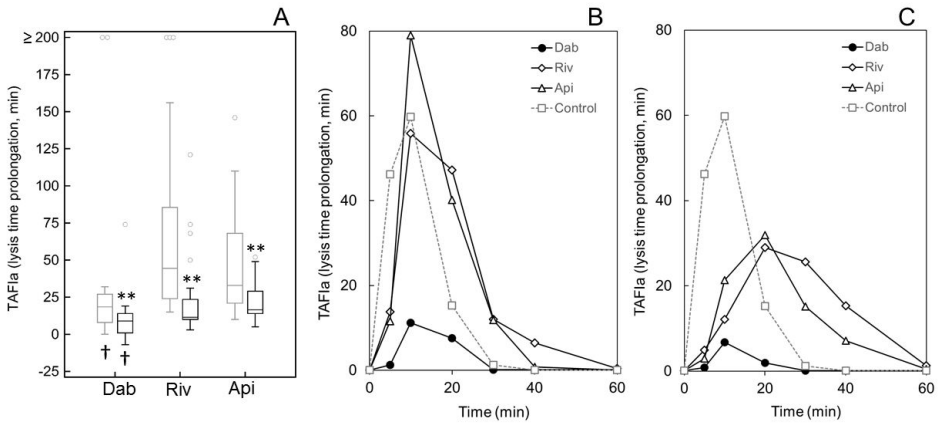

Fig. 3. TAFIa generation in plasma of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). Defibrinated plasma was challenged with low TF and TAFIa generation was assessed by a fibrinolytic assay (see methods for details). TAFIa activity generated in trough (grey boxes) and peak samples (black boxes) 10 min after clotting activation is illustrated in panel A. TAFIa generation curves in patients' samples taken at trough and peak are illustrated in panels B and C, respectively. Each point represents the geometric mean of six samples. The dotted line denotes pooled normal plasma. Mean concentrations of drugs in patient plasma samples showed in B and C were as follows: dabigatran, 62.4 and 178 ng/mL; rivaroxaban, 33.1 and 277 ng/mL, apixaban, 86.4 and 215 ng/mL. Results are expressed as PTCI-sensitive prolongation of lysis time. **, $P \leq 0.0001$ versus trough (Wilcoxon test); †, $P < 0.05$ versus corresponding samples of rivaroxaban and apixaban groups (Kruskal-Wallis test followed by pairwise comparison according to Conover).

Influence of thrombomodulin

Because direct thrombin inhibitors (DTIs) have been shown to inhibit fibrinolysis and enhance thrombin generation in vitro if relatively high concentrations of soluble TM were included in the assay system [5,14,15], we tested patients' samples in the presence of 4 nM TM. Under this condition, clot lysis time in dabigatran group was appreciably longer than in the other two groups both at peak and trough drug concentrations (Fig. 4A). The difference among groups became even greater when the antifibrinolytic activity of TM was calculated as TM-induced prolongation of lysis time (TM lysis ratio, Fig. 4B). Of note, there was a strong positive

correlation between dabigatran concentration and TM lysis ratio (Table 2), suggesting that dabigatran increased rather than decrease the fibrinolytic resistance. Moreover, TM lysis ratio was significantly higher in peak than in trough dabigatran samples. On the contrary, in rivaroxaban and apixaban groups, drug concentration did not influence TM lysis ratio. In the CAT assay, the addition of thrombomodulin to dabigatran samples reduced neither ETP nor peak thrombin levels (Fig. 5), suggesting that the drug made the plasma resistant to the anticoagulant activity of TM. On the contrary, TM addition to plasma from rivaroxaban and apixaban groups resulted in a marked reduction of both ETP and peak thrombin activity (Fig. 5).

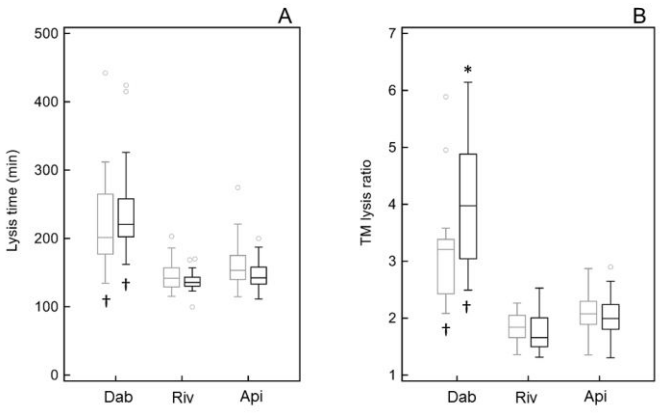

Fig. 4. Influence of thrombomodulin (TM) on fibrinolytic resistance of plasma of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). A, lysis times in the presence of 4 nM TM in trough (grey boxes) and peak samples (black boxes). B, lysis time prolongation induced by TM, calculated as TM lysis ratio (ratio of lysis times in the presence and in the absence of TM). *, P = 0.002 versus trough (Wilcoxon test); †, P < 0.05 versus corresponding samples of rivaroxaban and apixaban groups (Kruskal-Wallis test followed by pairwise comparison according to Conover).

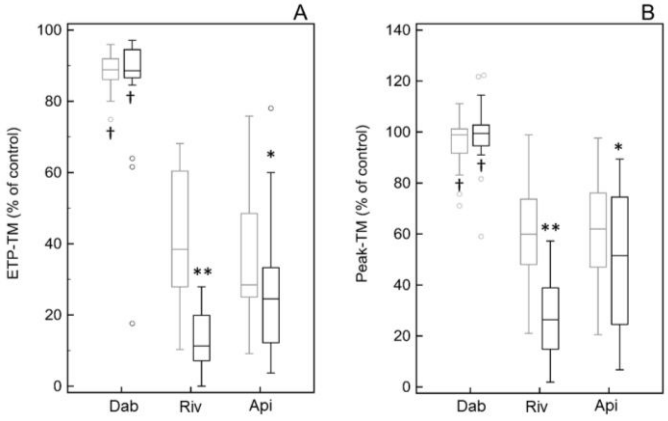

Fig. 5. Influence of thrombomodulin (TM) on thrombin generation in plasma of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). Thrombin generation was measured in trough (grey boxes) and peak samples (black boxes) by CAT. ETP (A) and peak thrombin (B) in the presence of TM (4 nM) is reported as percent of values in the absence of TM. **, P < 0.0001 and *, P < 0.05 versus trough (Wilcoxon test); †, P < 0.05 versus corresponding samples of rivaroxaban and apixaban groups (Kruskal-Wallis test followed by pairwise comparison according to Conover).

In vivo markers

Circulating levels of plasmin-antiplasmin complex were slightly but significantly higher in peak than in trough samples in all groups (Fig. 6B). On the contrary, prothrombin fragment 1+2 and TAFIa/ai did not differ between peak and trough samples in any group (Fig. 3A and C). Intergroup analysis revealed that PAP levels were higher in apixaban group whereas TAFIa/ai levels were lower in dabigatran group. None of the biomarkers showed a statistically significant correlation with drug levels with the exception of F1+2, which resulted significantly correlated with apixaban levels (Table 2).

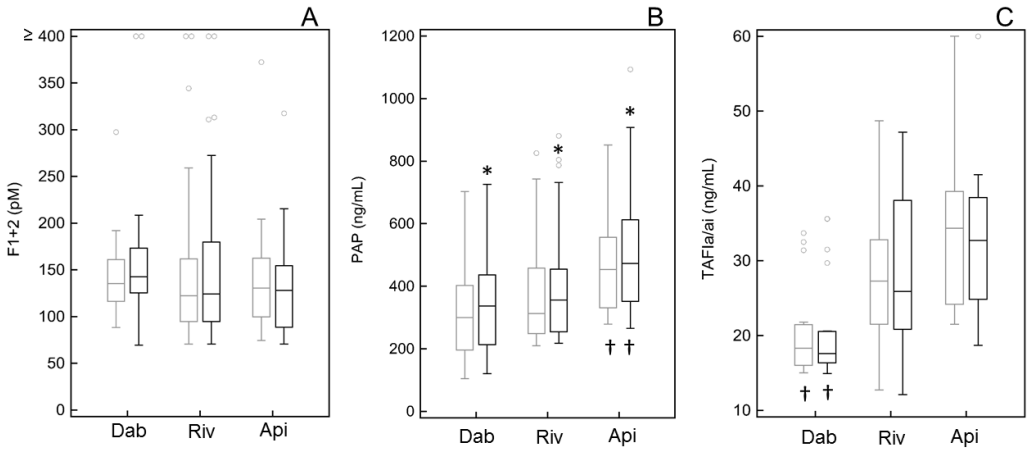

Fig. 6. Fibrinolysis and coagulation markers in plasma of patients treated with dabigatran (Dab), rivaroxaban (Riv) or apixaban (Api). A, prothrombin fragment F1+2; B, PAP complex; C, activated TAFI plus its inactive derivative (TAFIa/ai). Biomarkers were measured at trough (grey boxes) and peak (black boxes) by specific ELISAs. *, $P < 0.001$ versus trough; †, $P < 0.05$ versus corresponding samples of the other two groups (Kruskal-Wallis test followed by pairwise comparison according to Conover).

Discussion

Our study shows that dabigatran treatment reduces the fibrinolytic resistance of plasma clots whereas neither rivaroxaban nor apixaban influenced the fibrinolytic response to any noticeable extent. To investigate the impact of direct oral anticoagulants on fibrinolysis, we tested plasma samples at peak and trough drug levels, using an in vitro clot lysis model considered of pathophysiological relevance [23]. Two observations indicate that dabigatran administration reduces fibrinolytic resistance: 1) lysis time at peak was appreciably and significantly shorter than at trough; 2) lysis time was strongly and inversely correlated with dabigatran level, suggesting a concentration-dependent profibrinolytic effect of the drug. On the contrary, in patients treated with rivaroxaban or apixaban, the lysis time of plasma clots was similar in peak and trough samples and unrelated to drug level. The lack of effect on fibrinolysis is particularly surprising in rivaroxaban-treated patients, in whom the difference in drug concentration between peak and trough samples was greater than 7-fold. Another important observation supporting the profibrinolytic effect of dabigatran is that the lysis time of peak samples from dabigatran-treated patients was significantly shorter than the corresponding lysis time recorded in the other 2 groups.

The variable effect on fibrinolytic resistance is likely due to the different ability of anticoagulants to inhibit thrombin-mediated TAFI activation. Firstly, TAFIa activity generated after clotting activation was markedly lower in dabigatran than in rivaroxaban and apixaban samples at both trough and peak. Secondly, upon

neutralization of TAFIa by PTCI, the difference in lysis time between the 3 groups disappeared. Thirdly, the contribution of TAFI activation to fibrinolysis, measured as PTCI ratio, was significantly lower in dabigatran group, even when the trough samples were compared. Based on these findings and considering that TAFIa inhibits fibrinolysis with a threshold mechanism [14], it is plausible to hypothesize that in dabigatran samples the levels of TAFIa descend below the threshold concentration needed to halt the fibrinolytic process, whereas in rivaroxaban and apixaban groups they remain mostly above the threshold both at trough and peak drug concentrations (Fig. 7). In fact, looking at the TAFIa generation curves, it can be realized that the reduction in maximum TAFIa level seen in peak samples of rivaroxaban and apixaban groups is offset by a more sustained activation of TAFI. Unfortunately, we could not establish if the greater effect of dabigatran on TAFI activation was due to a greater inhibition of thrombin generation because the CAT parameters in dabigatran samples were calculated manually and expressed in arbitrary units due to the interference of the drug with the internal calibrator [22].

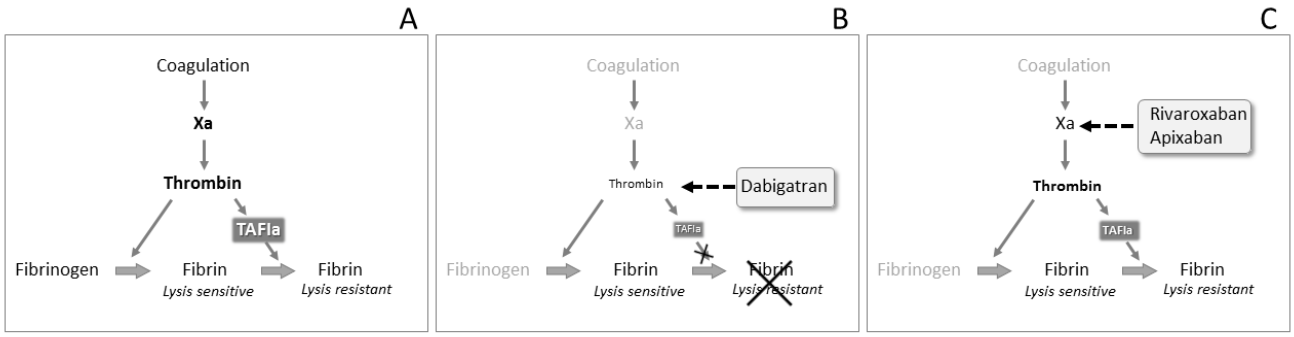

Fig. 7. **Hypothesis on the differential effect of anticoagulants on TAFI-mediated inhibition of fibrinolysis**. A, in the absence of anticoagulants, thrombin generation is high and sustained, leading to the generation of enough TAFIa to overcome the threshold level needed to halt fibrinolysis (through the conversion of fibrin from a lysis sensitive to a lysis resistant form). B, dabigatran administration markedly reduces thrombin-mediated TAFI activation so that the concentration of TAFIa is kept below the critical threshold level, resulting in faster fibrinolysis. C, rivaroxaban or apixaban treatment has a weaker effect on thrombin-dependent TAFI activation so that the levels of TAFIa remain mostly above the threshold level, resulting in fibrinolysis inhibition.

Anticoagulants have been reported to stimulate fibrinolysis through TAFI-dependent and TAFI-independent mechanisms [7,8]. In our clot lysis model, the effect of dabigatran was largely TAFI-mediated as suggested by the fact that the difference in lysis time between peak and trough samples became much smaller in the presence of PTCI. Actually, under this condition a small shortening of lysis time at peak was also observed with rivaroxaban, suggesting a weak TAFI-independent profibrinolytic effect.

Several in vitro and animal studies indicate that direct thrombin inhibitors, unlike anti-Xa inhibitors, enhance thrombin generation and make clots more resistant to fibrinolysis when tested in the presence of TM [5,14,16,17]. Given the potential implications of these findings, we evaluated if similar prothrombotic effects could be seen in clinical samples. TM addition to patients' plasma prolonged the lysis time of dabigatran

samples markedly more than in rivaroxaban and apixaban samples. In addition, TM-induced lysis time prolongation was greater in peak than in trough samples from dabigatran group and positively correlated with drug level, indicating that the higher the dabigatran concentration the more pronounced the antifibrinolytic effect of TM. In contrast, neither rivaroxaban nor apixaban treatment influenced TM-induced prolongation as indicated by the lack of difference in lysis time between trough and peak samples and by the lack of correlation between lysis time and drug level. Concerning thrombin generation, we found that TM addition had little or no effect on ETP and peak thrombin in dabigatran samples, whereas it appreciably reduced both CAT parameters in rivaroxaban and apixaban samples, indicating that dabigatran treatment induces a strong resistance to the anticoagulant effect of TM. As to the mechanisms behind these paradoxical effects of dabigatran, several studies suggest that the enhancement of thrombin generation is due to the blockade of protein C activation resulting from the inhibition of thrombin-TM by DTIs [16,17], whereas the inhibition of fibrinolysis was recently shown by our group to be protein C-independent [15]. If similar paradoxical effects would occur in vivo, dabigatran treatment might expose the patients to an enhanced risk of thromboembolic events, as suggested by some investigators to explain the increased incidence of myocardial infarction in patients under dabigatran [24]. However, the circulating levels of prothrombin F1+2 and PAP complex detected in our patients do not support the concept that dabigatran treatment increases thrombin generation or decreases plasmin formation. In fact, F1+2 levels in dabigatran-treated patients were in the same low range as in patients receiving the anti-Xa drugs. Moreover, PAP levels were similar in dabigatran and rivaroxaban groups and only slightly higher in apixaban group. Probably, the influence of TM on dabigatran activity in vivo is different from that seen in vitro because of the localization of TM on the vascular endothelial surface and the dynamic flow conditions (as opposed to soluble TM and static conditions in vitro). Nevertheless, considering that circulating F1+2 and PAP complex reflect thrombin and plasmin generation at systemic level under resting conditions, we cannot exclude that dabigatran administration might exert paradoxical prothrombotic effects locally upon clotting challenge. If so, the unwanted effects of the drug should likely be limited to small vessels, where the concentration of TM is high enough to affect the activity of direct thrombin inhibitors. Indeed, based on the estimated number of TM molecules per surface area [25], it was calculated that a TM concentration > 1 nM (i.e. the concentration needed to unmask the paradoxical prothrombotic effects of dabigatran) can be found in vessels with a diameter < 0.5 mm [5]. PAP levels were significantly higher at peak than at trough drug concentration in all three groups. However, the difference was small (mean increase < 10%) and PAP levels did not correlate with drug concentration in any groups, making it difficult to draw any conclusion about the influence of drugs on in vivo plasmin generation under resting conditions. As a matter of fact, also F1+2 levels were not or were poorly influenced by drugs. Previous studies showed that the levels of clotting markers during treatment with direct oral anticoagulants were reduced when compared to the levels measured prior to starting the anticoagulant

treatment [26-29]. However, consistent with our results, no difference in F1+2 levels was seen between samples collected at different intervals from rivaroxaban intake [30], suggesting that the low grade clotting activation under anticoagulant treatment is poorly sensitive to diurnal changes in drug concentration. Concerning in vivo TAFI activation, we did not find any difference in circulating TAFIa/ai between peak and trough samples of all groups. However, we did observe significantly lower levels of plasma TAFIa/ai in dabigatran group as compared to anti-Xa groups, maybe because dabigatran concentrations at trough and peak were able to inhibit TAFI activation by the low amount of circulating thrombin.

Thrombosis is a localized phenomenon, taking place in medium-large vessels, triggered by the aberrant expression of procoagulant factors (mainly tissue factor), which leads to the formation of enough thrombin to overwhelm the natural anticoagulants and cause fibrin formation [31]. Moreover, thrombin activates a number of mechanisms, among which TAFI activation, that make the nascent thrombus resistant to fibrinolysis [7,8]. Anticoagulant treatment is intended to prevent thrombus formation through the reduction of thrombin generation/activity. However, whether a reduced coagulability will lead to the formations of thrombi that are more susceptible to lysis depends on the intensity and duration of thrombin inhibition. This aspect is particularly relevant for TAFI-dependent inhibition of fibrinolysis because the reduction in TAFI activation resulting from less thrombin formation may be uninfluential if the TAFIa levels are still above the threshold concentration that inhibits the fibrinolytic process [14]. Our clot lysis model mimics to some extent the conditions encountered at site of thrombus formation such as the “expression” of TF and the “release” of t-PA. Therefore, based on our findings, it can be suggested that, at site of clotting challenge, dabigatran might offer an advantage over rivaroxaban and apixaban, being more efficient in down-regulating the TAFI-mediated inhibition of fibrinolysis.

Conclusions

Our data suggest that in patients with atrial fibrillation dabigatran treatment makes clots more susceptible to fibrinolysis mainly through the attenuation of thrombin-mediated activation of TAFI. Rather surprisingly, neither rivaroxaban nor apixaban treatment influenced the fibrinolytic capacity of plasma, contradicting in vitro data that rivaroxaban, at clinically relevant concentrations, did reduce the fibrinolytic resistance of blood and plasma clots [6]. Whether the profibrinolytic effect of dabigatran translates in a greater antithrombotic efficacy as compared to anti-Xa inhibitors remains to be established. Up to now, no head to head trials directly comparing the drugs against each other have been performed. Nevertheless, indirect comparison by network meta-analysis, based on the major clinical trials on direct oral anticoagulants, showed that dabigatran 150 mg bid was associated with significantly lower stroke and systemic embolism as compared to rivaroxaban [32,33].

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