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The Effect of Direct Oral Anticoagulants on Antithrombin Activity Testing Is Abolished by DOAC-Stop in Venous Thromboembolism Patients

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• **Context.**—Direct oral anticoagulants (DOACs) may cause falsely negative results of antithrombin (AT) deficiency screening.

Objective.—To evaluate the impact of DOAC-Stop, an agent reversing *in vitro* effects of DOACs, on AT testing in anticoagulated patients.

Design.—We assessed 130 venous thromboembolism patients aged 46.7 ± 13.5 years. Blood samples were collected 2 to 27 hours after DOAC intake from 49 patients on rivaroxaban, 54 on apixaban, and 27 on dabigatran. Antithrombin activity was assessed using the activated factor X (FXa)-based and the activated factor II (FIIa)-based method twice, before and after DOAC-Stop treatment, together with plasma DOAC levels using coagulometric assays.

Results.—The use of DOAC-Stop did not influence AT activity measured using the FIIa-based assay, whereas there was a marked decrease in AT activity determined using the FXa-based assay ($\Delta\text{AT} = 16.9\%$; 95% CI, 12.9%–

19.1%). The AT-FIIa assay revealed decreased AT level ($<79\%$) in all 10 (7.7%) genetically confirmed AT-deficient patients treated with rivaroxaban or apixaban ($n = 5$ each), whereas the AT-FXa assay showed decreased AT activity ($<83\%$) in 2 subjects on rivaroxaban and 1 on apixaban with low plasma DOAC concentrations (<90 ng/mL). After DOAC-Stop median AT-FXa activity lowered from 83.5% (interquartile range, 66%–143%) to 65.5% (interquartile range, 57%–75%; $P = .005$; $\Delta\text{AT} = 18\%$) in AT-deficient patients, without any falsely negative results. The ΔAT in the FXa-based assay correlated with rivaroxaban and apixaban concentrations in the AT-deficient patients ($r = 0.99$, $P < .001$).

Conclusions.—Application of DOAC-Stop enables reliable evaluation of AT deficiency screening in patients taking rivaroxaban or apixaban and tested using the FXa-based method.

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Antithrombin (AT) is the main human endogenous anticoagulant that primarily inactivates thrombin and activated factor X (FXa), and to a lesser extent activated factors XII, XI, and IX. Antithrombin has low variability in the general population, and its activity ranges from 80% to 120%. Antithrombin deficiency was first reported by Olav Egeberg¹ in 1965, and it is considered a potent thrombophilia, with 80% prevalence of thrombosis in the lifetime. Molecular analysis of the gene encoding AT (*SERPINC1*) on

chromosome 1q25.1 is recommended in subjects with AT activity lower than 70%, where the mutation rate is 90% or more.^{2,3} A meta-analysis of 35 studies showed that the odds ratio for the first venous thromboembolism (VTE) among individuals with AT deficiency is 14 (95% CI, 5.5–29) with an annual risk of 1.2% (95% CI, 0.8%–1.7%).⁴ Antithrombin deficiency is diagnosed in up to 5% of patients following VTE.⁴

Because in everyday practice AT activity is measured using bovine thrombin- or FXa inhibition-based tests,⁵ a false-negative detection of AT deficiency with the FXa inhibition-based test is observed in some patients treated with direct oral anticoagulants (DOACs).⁶ Thrombin or FXa inhibitors affect several coagulation tests, providing false-positive and false-negative results of prothrombin time, activated partial thromboplastin time, protein S and protein C levels, lupus anticoagulant, or activated protein C resistance.^{7–12} Therefore, to appropriately interpret results of routine coagulation assays in patients taking DOACs, drug removal agents based on activated carbon have been designed to eliminate their interference with clotting tests.¹³ In previous studies,^{14–16} DOAC removal agents, including to date DOAC-Stop and DOAC-Remove, have been shown to effectively reduce plasma DOAC concentrations, leading to appropriate results of thrombophilia screening tests in patients receiving DOACs.

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The aim of this study was to evaluate whether DOAC-Stop is useful for AT deficiency testing in VTE patients taking DOACs.

MATERIALS AND METHODS

Patients

We recruited 130 VTE patients receiving rivaroxaban, apixaban, or dabigatran for at least 3 months prior to enrollment, who according to recommendations should take the last dose of drug 12 hours (for dabigatran or apixaban) or 24 hours (for rivaroxaban) before the visit in the outpatient clinic. All patients were referred to the Center for Coagulation Disorders in the John Paul II Hospital, Krakow, Poland, from November 2018 to October 2019 for thrombophilia screening.

Exclusion criteria were acute infection, known malignancy, chronic kidney disease stage III through V (estimated glomerular filtration rate <60 mL/min/1.73 m²), severe liver injury, and pregnancy.

Demographic and clinical data were collected by a questionnaire. The Jagiellonian University Ethical Committee approved the study and all the participants provided their written informed consent in accordance with the Declaration of Helsinki.

Laboratory Investigations

Venous blood was drawn from the antecubital vein with minimal stasis between 8 AM and 1 PM. Blood samples were collected into citrated tubes (9:1 of 0.106 M sodium citrate; Monovette, Sarstedt, Nümbrecht, Germany), centrifuged at 2500g and 20°C for 20 minutes to obtain platelet-poor plasma, snap frozen within 30 minutes, and stored in aliquots at -80°C until analysis.

Creatinine levels were assessed using a routine laboratory assay. Prothrombin time and activated partial thromboplastin time were measured using a BCS-XP analyzer (Siemens Healthcare Diagnostics, Marburg, Germany). Fibrinogen was measured by the von Clauss method. Antithrombin activity was measured using an assay based on FXa inhibition (Innovance Antithrombin, Siemens Healthcare Diagnostics) or thrombin-inhibition assays (Siemens Healthcare Diagnostics); the reference ranges provided by the manufacturer were from 83% to 118% for AT-FXa and from 79% to 112% for activated factor II (FIIa).

Plasma was tested at baseline and following treatment with DOAC-Stop (the DOAC-Stop was kindly provided by Haematex Research, Sydney, Australia) according to the manufacturer's instructions. One tablet of DOAC-Stop (18 mg) was added to 1 mL of citrated plasma thawed at 37°C, mixed gently using a rotating shaker for 5 minutes, and centrifuged for 5 minutes at 2000g at room temperature. Plasma supernatants were carefully removed, avoiding resuspension of the precipitate, and used for the coagulation tests. Technicians were unaware of the results of other tests.

Rivaroxaban and apixaban concentrations were measured using the chromogenic Biophen DiXal test (Hyphen BioMed, Neuville-sur-Oise, France) with specific calibrators. Plasma concentrations of dabigatran were determined using the Hemoclot thrombin inhibitor assay (Hyphen BioMed) according to the manufacturer's instructions. Direct oral anticoagulant plasma concentrations ranging from 0 to 30 ng/mL are presented as less than 30 ng/mL.

Genetic Analysis

After obtaining the patients' informed written consent DNA was extracted from whole blood or a buffy coat according to the manufacturer's protocol, using Gene MATRIX Quick Blood DNA Purification Kit (Eurex, Gdansk, Poland) and stored at -80°C until analysis. The whole *SERPINC1* (NM_000 488.3) gene was analyzed as previously described.¹⁷ For all samples with no causative point mutation identified, screening for large deletion/insertion was performed by multiplex ligation-dependent probe amplification (SALSA MLPA kit for P227 SerpinC1, Holland, Amsterdam, The Netherlands).

Statistical Analysis

Continuous variables were expressed as mean \pm SD or median with interquartile range (IQR). Normality of the data was assessed using the Shapiro-Wilk test. Categorical variables were presented as numbers and percentages and were compared by 2-sided Pearson χ^2 or Fisher exact test. Differences between 2 groups were compared using the Student *t* test for normally distributed continuous variables, and for nonnormally distributed continuous variables the Mann-Whitney *U* test was used. For paired data, the Student *t* test or the Wilcoxon signed rank tests were used as appropriate. Associations between nonparametric or parametric variables were assessed by Spearman or Pearson tests, respectively. $P < .05$ was considered statistically significant. All statistical analyses were performed using STATISTICA software Version 12.5 (StatSoft, Kraków, Poland).

RESULTS

Patient Characteristics

A total of 130 VTE patients, including 49 patients (37.7%) taking rivaroxaban (15 mg/d in 4 subjects and 20 mg/d in the remainder), 54 (41.5%) on apixaban (2.5 mg twice daily in 14 and 5 mg twice daily in 40 subjects), and 27 (20.8%) on dabigatran (110 mg twice daily in 4 and 150 mg twice daily in 23 patients) were analyzed (Table 1). Initial median plasma concentrations of DOACs were as follows: 104 ng/mL (IQR, 45–334 ng/mL) for rivaroxaban, 93.5 ng/mL (IQR, 64–145) for apixaban, and 71 (48–144) ng/mL for dabigatran. Median AT activity in the FIIa-based assay was 102% (IQR, 94%–109%), whereas for the FXa-based method AT activity was 130% (IQR, 117%–147%).

Among enrolled individuals, 37 patients (28.5%) on rivaroxaban, 44 (33.8%) on apixaban, and 26 (20%) on dabigatran did not follow the recommendation regarding the suggested time since DOAC intake before the visit. There were no differences in demographic, clinical, or laboratory variables among patients treated with rivaroxaban, apixaban, or dabigatran (Table 1). Antithrombin activity measured by the FXa-based method correlated with plasma concentrations of rivaroxaban ($r = 0.57$, $P < .001$) and apixaban ($r = 0.30$, $P = .047$), whereas dabigatran concentrations correlated with AT measured by the FIIa-based assay ($r = 0.45$, $P = .03$). The time since the last dose of rivaroxaban, apixaban, or dabigatran did not correlate with AT activity measured by the FIIa- or FXa-based assays.

AT Activity at Baseline

Genetically confirmed AT deficiency was found in 10 patients (7.7%) on rivaroxaban ($n = 5$) and apixaban ($n = 5$), including 4 patients with type I and 6 patients with type II AT deficiency (Table 2). All patients had decreased AT activity assessed using the FIIa-based method, but 3 AT-deficient patients had decreased AT activity using the FXa-based assay (2 subjects on rivaroxaban with drug concentrations equal to 30 and 31 ng/mL and 1 on apixaban, drug concentration 89 ng/mL) (Table 2). With the FXa-based AT assay, 7 AT-deficient patients (5.4%), including 3 patients (2.3%) on rivaroxaban and 4 (3.1%) on apixaban, had AT activity above the cutoff value of 83% or higher. There were no AT-deficient patients taking dabigatran. Of note, in 3 individuals with decreased AT activity both assays showed similar results (median difference 1% [minimum 1%, maximum 8%]) (Table 2). We found no differences in AT activity between subjects with type I and type II AT deficiency (Table 2). In AT-deficient patients, AT activity measured by the FXa- but not the FIIa-based method was

Variable	All patients (N = 130)	Patients on Rivaroxaban (n = 49)	Patients on Apixaban (n = 54)	Patients on Dabigatran (n = 27)	P
Age, mean ± SD, y	46.7 ± 13.5	49.3 ± 14.5	42.7 ± 12.8	50.0 ± 12.0	.39
Male, No. (%)	49 (37.7)	19 (38.8)	20 (37.0)	10 (37.1)	.98
Body mass index, mean ± SD, kg/m ²	27.5 ± 4.3	27.0 ± 4.7	27.0 ± 4.4	28.0 ± 3.4	.54
Laboratory investigations					
Activated partial thromboplastin time, median (IQR), s	44.7 ± 10.2	...
Prothrombin time, median (IQR), s	...	13.1 (11.9–14.7)	11.7 (11.4–12.3)
AT activity, median (IQR), %					
FIIa assay	102 (94–109)	104 (85–110)	99.5 (94–107)	106 (95–115)	.14
FXa assay	130 (117–147)	143 (126–158)	130.5 (118–142)	108 (104–117)	<.001
Fibrinogen, mean ± SD, mg/dL	310 ± 70	320 ± 80	300 ± 70	310 ± 60	.61
Creatinine, median (IQR), mg/dL	0.84 (0.74–0.96)	0.81 (0.74–0.97)	0.78 (0.71–0.89)	0.85 (0.78–1.01)	.06
eGFR, median (IQR), mL/min/1.73 m ²	92 (84–105)	87 (77–102)	95 (89–109.5)	94 (86–105)	.03
Plasma DOAC concentration, median (IQR), ng/mL	...	104 (45–334)	93.5 (64–145)	71 (48–144)	...
Declared time since the last DOAC intake, median (IQR), h	5.25 (3.5–14.75)	5.5 (3.5–22)	5.5 (3–13)	5 (4–6)	.48
Thrombophilia screening, No. (%)					
Antiphospholipid syndrome	11 (8.5)	4 (8.2)	5 (9.3)	2 (7.4)	>.99
Factor V Leiden G>A	16 (12.3)	7 (14.3)	6 (11.1)	3 (11.1)	.94
Prothrombin 20210 G>A	1 (0.8)	1 (2.0)	0	0	>.99
AT deficiency	10 (7.7)	5 (10.2)	5 (9.3)	0	>.99

Abbreviations: AT, antithrombin; eGFR, estimated glomerular filtration rate; FIIa, activated factor II; FXa, activated factor X; IQR, interquartile range.

positively associated with plasma rivaroxaban and apixaban concentrations ($r = 0.44$, $P < .001$). The declared time since the last intake of rivaroxaban or apixaban did not correlate at baseline with the AT activity measured by the FIIa- ($P = .49$) or FXa-based ($P = .40$) assays in AT-deficient patients.

AT Activity Post-DOAC-Stop

Removal of DOACs using DOAC-Stop did not influence AT activity measured using the FIIa-based assay in the whole patient group (Table 1). A change in AT activity between the initial value and that obtained after DOAC-Stop was negligible (Δ AT 1.18%; 95% CI, -0.03% to 2.03% ; Figure 1). In contrast, we found a marked decrease in AT activity determined using the FXa-based assay following the

DOAC-Stop (Δ AT = 16.9%; 95% CI, 12.9%–19.1%; Table 1; Figure 2).

DOAC-Stop treatment resulted in an increased number of patients with AT activity lower than 83% in the FXa-based assay, from 3 to 10 subjects (Table 2), which means a correct identification of the 7 falsely negative patients at baseline as AT deficient. In the FXa-based method, Δ AT calculated for the subgroup of AT-deficient patients was 18% (95% CI, 9.5%–40.5%; median AT activity at baseline, 83.5%; IQR, 66%–143%; versus median AT activity after DOAC-Stop 65.5%; IQR, 57%–75%; $P = .005$). Δ AT calculated based on the results of the FXa assay was associated with rivaroxaban and apixaban concentrations in the AT-deficient patients ($r = 0.99$, $P < .001$).

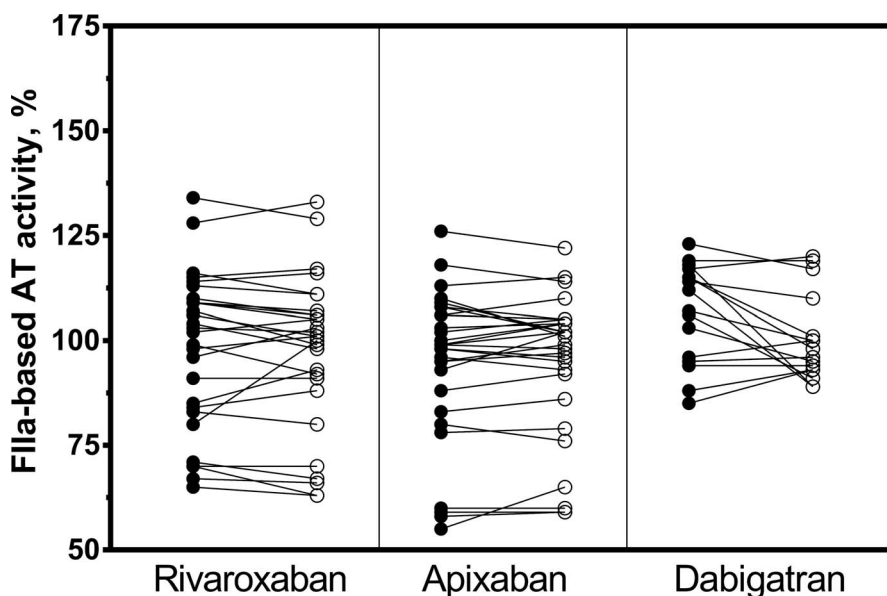


Figure 1. Antithrombin (AT) activity assessed using activated factor II (FIIa)-based method in patients taking direct oral anticoagulants at baseline (filled circles) and after DOAC-Stop application (open circles).

DISCUSSION

To our knowledge, this study is the first to show that DOAC-Stop is helpful in AT-deficiency testing in patients taking DOACs. Interpretation of AT testing results in patients on DOACs is challenging because DOACs can overestimate AT activity in patients with this inherited thrombophilia.⁶ Such overestimation may lead to misclassification of AT-deficient patients as individuals free of this thrombophilia.¹⁸ The FXa-based AT activity assay contains a chromogenic substrate, which is converted by the excess of exogenous FXa (not inhibited by AT in the presence of heparin) into 2 products, 1 of which can be quantified by the coagulation analyzer. The amount of the chromogenic product generated is inversely proportional to the AT activity in the tested sample. Thus, if FXa inhibitors such as rivaroxaban or apixaban are present in the plasma sample, FXa is blocked and AT activity overestimated. The FXa-based AT assays are unaffected by the presence of a thrombin inhibitor, dabigatran.¹⁹

Our findings increase the current knowledge on the optimal diagnostic workup of ambulatory VTE patients treated with DOACs and suspected of inherited thrombophilia. A widespread use of DOAC-Stop or other agents of similar activity should be available in tertiary centers dealing with thrombophilia testing to facilitate AT assessment in everyday practice.

Some experts recommend performing genetic analysis of the *SERPINC1* gene in patients with AT activity below 75%²⁰ and patients with AT activity between 75% and 83% are not classified as AT deficient, and further diagnostic assessment of this subgroup is individualized. However, detrimental mutations could be detected also in patients with AT levels above 75%, which might be important for their prognosis and genetic counseling.^{5,21}

In our cohort, we found that 4 AT-deficient patients on DOACs had a borderline AT activity between 83% and 86% in the FXa-based assay and that DOAC-Stop reduced those values to 61% to 75%.

The impact of DOACs on AT activity in patients with different genetic causes of AT deficiency is unclear, and the current findings provide some interesting observations. More than 250 mutations have been described in the AT gene, including type I deficiencies with reduced AT functional activity and antigen levels and type II with reduced activity at normal AT antigen levels.^{4,22} In contrast to type I AT deficiencies, type II deficiencies are related to alterations of the reactive site of AT (type IIRS), heparin-binding site (type IIHBS), or pleiotropic effects (type IIPE).¹⁷ Screening of AT deficiency is based in the first line on chromogenic functional assays, which measure the inhibition of FIIa or FXa in the presence of heparin as a cofactor.²³ Antithrombin activity assessed without heparin is useful to determine type IIHBS.²⁴ Of note, AT activity measured using the FIIa-based assay in type IIHBS heterozygote patients is mostly within the reference range, but is significantly reduced using the FXa-based assay, which is characteristic of patients with the p.Leu99Phe mutation (AT Budapest 3) or p.Pro41Leu (AT Basel).²⁵ Therefore, it has been suggested that the FXa-based assay is the method of choice for diagnosis of type IIHBS AT-deficient patients.²⁶ We confirmed that treatment with rivaroxaban and apixaban overestimates AT activity measured by FXa- but not FIIa-based assay^{6,7} in AT-deficient individuals. We found that DOAC-Stop might provide reliable results of AT deficiency

Table 2. Effects of DOAC-Stop on Antithrombin Activity in 10 Patients With Genetically Confirmed Antithrombin Deficiency

Patient ID	Age, y/sex	DOAC	Plasma DOAC Concentration		Antithrombin Activity at Baseline, %		Antithrombin Activity After DOAC-Stop, %		Post-DOAC-Stop Change in Antithrombin Activity Using FXa-based method (Δ%)	Type of AT Deficiency	<i>SERPINC1</i> Gene Mutation
			Before DOAC-Stop, ng/mL	After DOAC-Stop, ng/mL	FIIa-Based Method	FXa-Based Method	FIIa-Based Method	FXa-Based Method			
1	50/M	Rivaroxaban	458	53	65	143	65	63	78	II	c.1157T>C p.Ile386Thr ^a
2	46/M	Rivaroxaban	311	<30	71	126	66	67	60	II	c.1157T>C p.Ile386Thr ^a
3	22/F	Apixaban	261	<30	60	107	63	60	44	I	c.625-2A>G ^b
4	45/M	Apixaban	112	<30	59	83	61	59	22	I	c.625-2A>G ^b
5	24/F	Rivaroxaban	102	<30	70	84	68	70	16	II	c.1157T>C p.Ile386Thr ^a
6	54/M	Apixaban	98	<30	78	86	75	79	11	II	c.1157T>C p.Ile386Thr ^a
7	44/F	Apixaban	98	<30	66	83	63	65	20	I	c.625-2A>G ^b
8	31/F	Apixaban	89	<30	58	66	59	57	9	I	c.948delC p.Leu317Cysfs*11 (not reported)
9	35/F	Rivaroxaban	31	<30	67	68	66	66	2	II	c.1157T>C p.Ile386Thr ^a
10	38/M	Rivaroxaban	30	<30	70	71	69	70	1	II	c.1157T>C p.Ile386Thr ^a

Abbreviations: DOAC, direct oral anticoagulant; FIIa, activated factor II; FXa, activated factor X; F, female; M, male.

^a Data from Di Minno et al.³⁶

^b Data from Wypasek et al.⁵

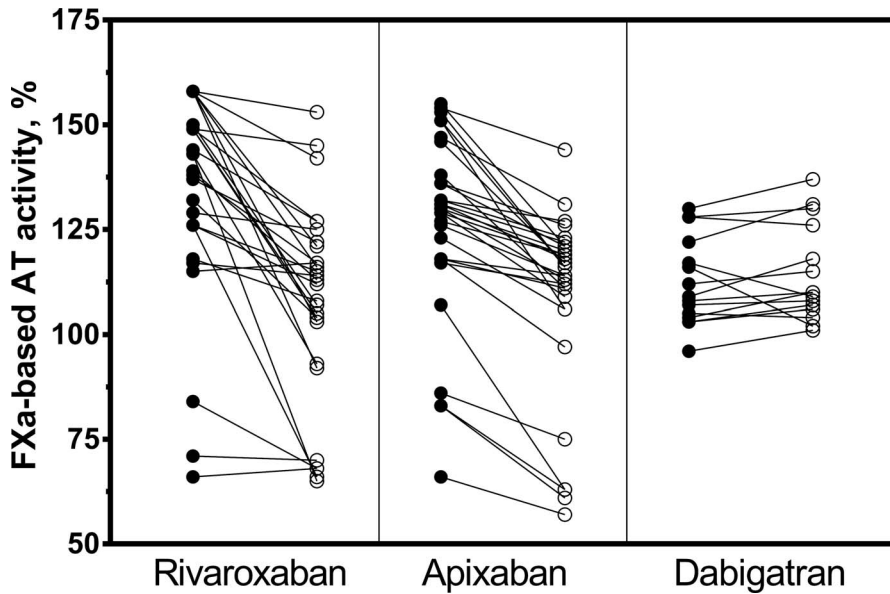


Figure 2. Antithrombin (AT) activity assessed using factor (F) Xa-based method in patients taking direct oral anticoagulants (DOACs) at baseline (filled circles) and after DOAC-Stop application (open circles).

screening in patients with DOAC concentrations above the clinically relevant level of 30 ng/mL²⁷ and even at peak DOAC concentrations, which in our study were 468 ng/mL for rivaroxaban and 261 ng/mL for apixaban. On the other hand, AT activity may be overestimated in patients on dabigatran if tested using the FIIa-based assay, especially at concentrations higher than 100 ng/mL according to the in vitro study by Douxfils et al.²⁸ Our study showed that dabigatran at median concentration of 71 ng/mL did not influence the results of AT activity assessed using both FXa- and FIIa-based assays in non-AT-deficient patients, which is in line with the previous study.¹⁴ In our cohort none of the patients on dabigatran had AT deficiency; thus, we did not resolve whether dabigatran may result in false-negative results of AT-deficiency screening and if DOAC-Stop is able to eliminate this interference.

From a practical point of view, the issue of the interval from the last DOAC dose to blood collection for AT measurement in patients on DOAC is important. For thrombophilia screening in patients taking DOACs it is recommended to perform laboratory investigations at least 12 hours after discontinuation of rivaroxaban and apixaban or 24 hours after discontinuation of dabigatran.^{29,30} However, longer discontinuation of DOACs in patients with high thrombotic risk can be dangerous and should be avoided. First, in this group of patients a preferred AT activity assay should be used. Direct oral anticoagulants have either FIIa- or FXa-inhibiting functions and may therefore interfere with AT activity tests that are based on the same principles.³¹ The FIIa-based assay is preferred in individuals treated with rivaroxaban and apixaban,³¹ excluding patients suspected of type IIHBS AT deficiency, in whom the FXa-based method should be performed.²⁶ Second, if the only available method for AT activity evaluation is the FXa-based assay, the DOAC-Stop can facilitate obtaining reliable results in patients on rivaroxaban and apixaban, especially when higher drug concentrations are detected. Moreover, our study showed that there is a discrepancy between declared time since last dose of DOAC intake and the actual DOAC plasma concentration, which in AT-deficient patients significantly influenced the results of AT deficiency screen-

ing by yielding false-negative results. This effect was abolished by the DOAC-Stop procedure.

This study has several limitations. First, the sample size was limited; however, we enrolled VTE patients from the outpatient clinic, reflecting a real-life setting.³² Second, the subgroup of patients taking dabigatran was small and none of the subjects with AT deficiency were treated with this agent, which is uncommonly used on a long-term basis in VTE patients nowadays, especially among those with severe inherited thrombophilia.^{33,34} Therefore, it remains unknown whether DOAC-Stop is able to provide appropriate results of AT-deficiency screening in patients taking dabigatran.³⁵ Third, in our study no patients with type IIHBS AT deficiency were identified; thus, the effectiveness of DOAC-Stop in this subgroup needs further investigation.

Our study showed that application of DOAC-Stop can be used in patients screened for AT deficiency while taking rivaroxaban or apixaban and tested using the FXa-based method and that if possible in such individuals AT activity should be assessed by FIIa-based assay. Further studies are needed to precisely evaluate the utility of DOAC-Stop and other similar reversal agents in patients with very high DOAC concentrations and those with diverse types of *SERPINC1* mutations. From a practical point of view, our findings might help performance of thrombophilia testing in everyday practice in patients who have taken a DOAC a few hours prior to blood draw, though the optimal strategy is to collect blood samples at least 24 hours after the last dose of a DOAC.

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