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ORIGINAL ARTICLE

Drug levels and bleeding complications in atrial fibrillation patients treated with direct oral anticoagulants

Sophie Testa[1] | Cristina Legnani[2] | Emilia Antonucci[2] | Oriana Paoletti[1] | Claudia Dellanoce[1] | Benilde Cosmi[3] | Vittorio Pengo[4] | Daniela Poli[5] | Rossella Morandini[1] | Roberto Testa[6] | Armando Tripodi[7] | Gualtiero Palareti[2] | Coordinator of START2-Register

[1]Haemostasis and Thrombosis Center, Ospedale di Cremona, Cremona, Italy

[2]Arianna Anticoagulazione Foundation, Bologna, Italy

[3]Angiology and Blood Coagulation, University Hospital of Bologna, Bologna, Italy

[4]Department of Cardiac, Thoracic and Vascular Sciences, Cardiology Clinic, Thrombosis Centre, University of Padua, Padova, Italy

[5]Thrombosis Centre, AOU Careggi, Firenze, Italy

[6]Clinical Laboratory and Molecular Diagnosis, INRCA-IRCCS National Institute, Ancona, Italy

[7]Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, IRCCS CàGranda Maggiore Hospital Foundation, Milano, Italy

Correspondence
Sophie Testa, Hemostasis and Thrombosis Center, Ospedale di Cremona, Viale Concordia 1, 26100 Cremona, Italy.
Email: s.testa@asst-cremona.it

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Abstract

Background: Direct oral anticoagulants (DOACs) are administered at fixed dose. The aim of the study was to evaluate the relationship between DOAC C-trough or C-peak plasma levels and bleeding complications in patients with non-valvular atrial fibrillation (NVAF).

Methods: Five hundred sixty five consecutive naive NVAF patients were enrolled. The DOAC measurements at C-trough and at C-peak (available in 411 patients) were performed at steady state, within the first month of treatment. Major bleeding (MB), clinically relevant non-major bleeding (CRNMB), and minor bleeding (MinB), occurring during 1 year of follow-up after blood sampling, were recorded. For each DOAC, interval of C-trough and C-peak levels was subdivided into four equal classes and results were attributed to these classes; the median values of results were also calculated.

Results: Two hundred eight patients were on apixaban, 185 on dabigatran, and 172 on rivaroxaban. For 1-[qqqdeletezzz] year follow up for all patients, we observed: 19 MB (3.36%), 6 CRNMB (1.06%), and 47 MinB (8.31%). The prevalence of bleeding patients with anticoagulant levels in the upper classes of C-peak activity (II + III + IV) was higher than that in the lowest class. Normalized results of C-peak levels were higher in patients with bleeding than in those without bleeding.

Conclusions: Bleeding complications during DOAC treatment were more frequent among atrial fibrillation (AF) patients with higher C-peak anticoagulant levels. In addition to a previous study that showed an increased risk of thrombotic complications in the patients with low C-trough levels, this study seems to indicate that patients with NVAF on DOACs would need a more accurate definition of their optimal therapeutic window.

KEYWORDS
atrial fibrillation, bleeds, direct oral anticoagulant, level, peak

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1 | INTRODUCTION

Currently, DOACs represent the first-line treatment in two clinical conditions: the prevention of stroke and systemic embolism in patients with NVAF and the treatment/prevention of venous thromboembolism.[1]

Available DOACs for NVAF and venous thromboembolism include dabigatran, a selective anti-factor IIa molecule, and three direct anti-factor Xa inhibitors: apixaban, edoxaban, and rivaroxaban. The DOACs' pharmacological characteristics, together with the assumed predictable dose-response, led to the indication of fixed dose administration without dose adjustment based on laboratory testing,[1] the latter being recommended only in special situations.[1]

The choice of DOAC dosage is based on the evaluation of clinical indications (NVAF, venous thromboembolism), patient characteristics (age, gender, body weight, concomitant administration of potentially interfering drugs), and renal and liver function, assuming that drug anticoagulant effect is prevalently controlled by these conditions.

Nevertheless, a high interindividual variability in the drug blood levels was shown with all DOACs, and post hoc analyses of phase III trials showed an association between DOAC plasma levels and thrombotic and bleeding complications during follow up.[2–9] Moreover, phase IV clinical trials have shown a higher interindividual variability if compared with phase III studies, confirming that real world patients differ from the selected populations enrolled in randomized trials.[5–9]Recently, we reported results of an observational study showing a relationship between low C-trough DOAC levels and the occurrence of thrombotic events in NVAF patients, particularly in patients with higher cardiovascular risk. That study supported the concept of assessing the anticoagulant DOAC levels at the C-trough steady state as a tool to optimize dosages of anticoagulation in NVAF patients.[10,11]

In the present study we analyzed the same patient population aiming at assessing a possible relationship between DOAC C-trough and/or C-peak levels, measured at steady state within the first month of treatment, and the bleeding events occurring during 1-year follow up after the day of blood sampling.

Essentials

- Currently, DOACs are given at fixed doses and do not require laboratory monitoring.
- Direct oral anticoagulant-specific measurements were performed at trough and peak.
- Patients who developed bleeding events showed higher DOAC plasma levels at peak.
- This study suggests the need of a more accurate DOAC dose assessment.

2 | METHODS

2.1 | Patients

This study was performed within the frame of activity of the START Laboratory Register, a branch of the START2-Register (Survey on anTicoagulatedpAtientsRegisTer) (NCT 02219984), supported by the Arianna Anticoagulazione Foundation (Bologna, Italy).[12] The study is an observational, multicenter study in patients with NVAF treated with dabigatran, rivaroxaban, or apixaban. It was conducted in four Anticoagulation Clinics (Ancona, Bologna, Cremona, Padua) affiliated with the Italian Federation of Anticoagulation Clinics (FCSA) and participating in the START2-Register. The DOACs have been introduced in Italy at different time from June 2013; during the period of the patients' enrolment the drugs available and reimbursed by the national health system were dabigatran, rivaroxaban, and apixaban.

The criteria for inclusion in the study and the characteristics of the investigated patient population (565 consecutive naive patients with NVAF) are detailed elsewhere.[13]

Patients were treated with any of the DOACs at a dosage based on clinical characteristics, at the discretion of the attending physician and according with the recommendations issued by the Italian regulatory agency. A total of 185 patients were on dabigatran (82 and 103 taking 150 mg or 110 mg twice daily, respectively), 172 on rivaroxaban (100 and 72 taking 20 mg or 15 mg once daily, respectively), and 208 on apixaban (154 and 54 taking 5 mg or 2.5 mg twice daily, respectively).

Baseline characteristics (demographic, clinical, risk factors, CHA_2DS_2-VASc Score, HAS-BLED, weight, body mass index, kidney and liver function, concomitant medications) were recorded in a structured database. Follow-up, as defined by FCSA guidelines, included clinical evaluation within the first month and every 3 months for 1 year. Patients' compliance and adherence to anticoagulant treatment were evaluated by manual pill counting at each visit. The persistence in the treatment was checked at the controls every 3 months and at the end of 1-year follow-up.

All bleeding and thromboembolic complications were recorded for 1 year of follow-up,which started the day of blood sampling and lasted 1 year, or less if the drug dosing was changed, or treatment was stopped, or switched to a different drug, or major bleeding or thrombotic events occurred. Results on thromboembolic complications had already been reported.[13] In this study, we report data on the relationship between the measured DOAC anticoagulant levels and the bleeding complications occurring during follow-up.

The bleeding events considered for the study includedMB, CRNMB, and MinB. For MB, the criteria reported by the International Society on Thrombosis and Haemostasis were adopted, including fatal bleeding or symptomatic bleeding in critical area or organ (i.e., intracranial, intraspinal, intraocular, pericardial, intraarticular, intramuscular with compartment syndrome, retroperitoneal) or bleeding causing a fall of hemoglobin level of 20 g/L (1.24 mmol/L or 2 g/dL) or more, or leading to transfusion of ≥2 units of whole blood or

red cells.[14] The CRNMB was defined as any overt bleeding requiring a medical intervention (hospitalization, surgery or interventional procedure, further diagnostic imaging, laboratory test, or specialist evaluation) and/or treatment discontinuation, and not meeting any of the criteria for major bleeding.[15] Minor bleeding was defined as any overt bleeding that was reported by the patients to the anticoagulation clinic and that did not require medical interventions.

All bleeding events were adjudicated by the local investigators on the basis of clinical signs and symptoms combined with objectively confirmed diagnostic radiology (magnetic resonance imaging, computed tomography, ultrasound investigation) or laboratory tests.

2.2 | Laboratory assays

Blood samples for 565 patients were collected within the first 15 to 25 days of treatment. Trough samples were obtained at 12 h from the last dose intake for dabigatran and apixaban, and at 24 h for rivaroxaban. Peak samples were collected in the same morning of trough samples; after trough sampling, patients had breakfast and assumed the drug; then waited 2 h in the outpatient clinic or went back to it to have peak sampling. However, some patients refused to wait in the outpatient clinic or to return to it in the same morning to have the second blood sampling for C-peak; in most cases they cited personal or family commitments for the refusal. This was the reason why only 411 peak samples were available for the study.

Blood samples were collected in vacuum plastic tubes (Vacutainer, Becton Dickinson, Plymouth, UK), containing 3.2% trisodium citrate (9:1 vol/vol, blood/anticoagulant). Tubes were centrifuged within 1 h from collection at 2000 g for 20 min and plasma was quickly frozen and stored at −80°C until testing. The DOAC levels, expressed as drug concentration-equivalent (ng/mL), were measured using commercial specific coagulation tests that, when compared with liquid chromatography tandem mass spectrometry, have previously demonstrated good performance.[16–18]

Diluted thrombin time or anti-FIIa, calibrated for dabigatran, and specific anti-FXa assays calibrated for apixaban and rivaroxaban[16–18] were used to measure DOAC plasma levels. All tests were performed locally, within 3 months of plasma collection, using Stago (Asnieres-sur-Seine, France), Hyphen (Neuilly-sur Oise, France), and Siemens (Marburg, Germany) reagents on STA R (Stago, France) and CA 7000 (Siemens, Germany), according to manufacturer's indications as previously described.[9]

The limits of quantification (LOQ), as reported elsewhere,[13] were evaluated retesting a pooled normal plasma 10 times with each assay. Raw data, expressed as seconds or OD/min (Y1, Y2, ...),were used to calculate the standard deviation (SD). Then, raw data were transformed as follows: Y1′ = Y1 + 10 SD (Y2′ = Y2 + 10 SD, ...) or Y1′ = Y1 − 10 SD (Y2′ = Y2 − 10 SD, ...) for clotting and chromogenic assays, respectively. Each of the transformed raw data (Y1′, Y2′, ...) was used to calculate the drug concentration (ng/mL) on the calibration curves (X1, X2, ...). The mean value of X1, X2, ... was used as LOQ for each drug. Measured DOAC concentrations below LOQ were substituted with the LOQ values. The interval obtained for each test from the LOQ to the highest measurement value was divided into four equal interval classes and the patient results were distributed among these classes, ranging from that with the lowest (class I) to that with the highest levels (class IV).

The individual measured DOAC levels were also analyzed in relation to the expected plasma concentrations after therapeutic doses for each drug, as reported by Douxfils et al[19]; the results were then distributed among the high, normal, and low responders (above, within, or below the expected concentrations, respectively).

2.3 | Statistical analysis

Descriptive analysis was performed. Continuous variables are expressed as mean and SD or median and range. Categorical variables are expressed as frequencies and percentages. The incidence of bleeding events was calculated. Preliminary statistical analysis was performed using the Wilcoxon signed rank test (continuous variables) or the Fisher exact test (categorical data). The nonparametric Mann-Whitney *U* test was used for comparison between patients with and without bleeding events.

Due to the different ranges of plasma concentration of the three DOACs, a normalization of values was performed. Median value of each drug was calculated, and C-peak values were divided by the median value calculated for each DOAC.

Crude odds ratio and 95% confidence interval were calculated with logistic regression analysis to estimate the relative risk for bleeding events. Multivariate analysis by unconditional logistic regression was used to adjust for all possible confounding variables (performed for characteristics with a *P* value [qqq]=< .1 at univariate analysis). The SPSS software for Windows, version 22 (SPSS Inc, Chicago, IL) was used for data processing.

2.4 | Ethics

The study protocol of the START-Registry was approved by the local ethics committees and was conducted in accordance with the Declaration of Helsinki.

3 | RESULTS

The flowchart of the study and of investigated patients is shown in Figure 1. The main clinical characteristics of patients, detailed for each DOAC used, are shown in Table 1.

Median age was 80 years and was not different among patients treated with thethree drugs. Males were 315 (55.7%). Median CrCl was 69.0 (33 to 149). All patients showed normal liver function, as estimated by aspartate transaminase and alanine transaminase. Median values of CHA_2DS_2-VASc and HAS-BLED scores were not significantly different among the patients treated with the three drugs. Adherence, evaluated through the manual pill counting, was high, with an agreement between consumed and expected pills greater than 90% for the three drugs.

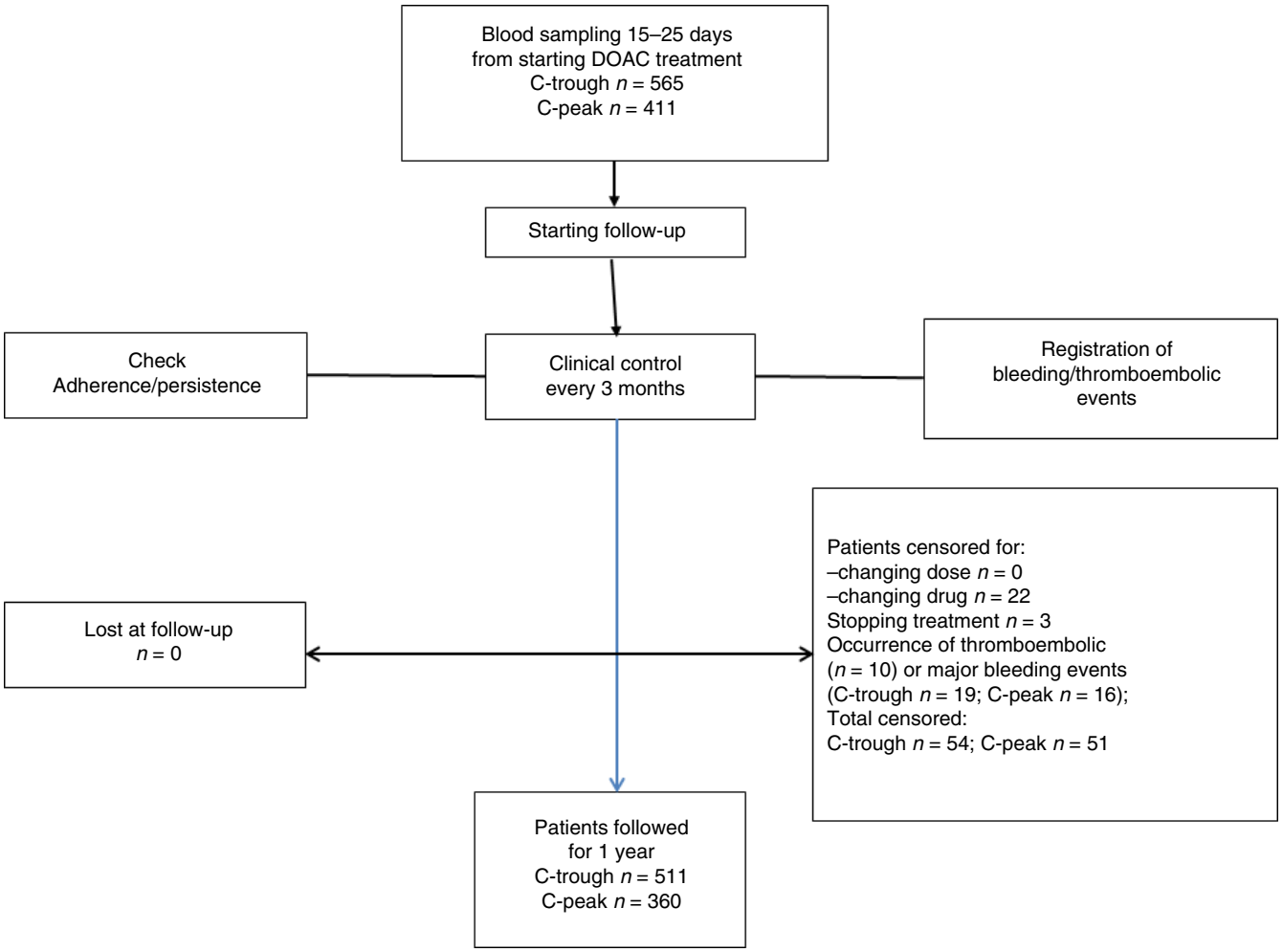

FIGURE 1 Flowchart of the study and included patients

TABLE 1 Main clinical characteristics of patients; results are reported as median (min-max)

	Dabigatran	Rivaroxaban	Apixaban	Total
Patients (n)	185	172	208	565
Age (years)	78 (44-94)	82 (57-97)	80 (49-94)	80 (44-97)
Gender (M/F)	105/80	95/77	115/93	315/250
BMI	26.9 (17.4-43.3)	25.5 (16.6-34.7)	26.2 (16.4-40.1)	26.2 (16.4-43.3)
Drug daily dose (no. of patients)	2 × 150 mg (82)	20 mg (100)	2 × 5 mg (154)	—
	2 × 110 mg (103)	15 mg (72)	2 × 2.5 mg (54)	
Creatinine clearance (mL/min/1.73 m^2)	70.5 (39-149)	66.5 (36-117)	69.0 (33-117)	69.0 (33-149)
CHA_2DS_2VASc	3 (0-7)	3 (0-7)	3 (0-9)	3 (0-9)
Mean ± SD	3.5 ± 1.5	3.0 ± 1.4	2.9 ± 1.3	3.15 ± 1.4
HASBLED	3 (0-7)	3 (0-7)	3 (0-9)	3 (0-9)
Mean ± SD	2.7 ± 1.1	2.4 ± 1.1	3.0 ± 1.1	2.7 ± 1.2

Note: Results are reported as median (min-max).
Abbreviations: BMI, body mass index.

During 1-year follow up, the following bleeding events were observed: 19 MB (3.4%), 6 CRNMB (1.1%), and 47 MinB (8.3%); however, for only 52 were measured C-peak results available (Table 2). No significant differences for patient characteristics were recorded between those with or without bleeding complications. Twenty percent of the bleeding events occurred within the first 3 months of

TABLE 2 DOAC anticoagulant levels measured at C-trough and C-peak; types and sites of bleeding events occurring during 1 year of follow-up

	C-Trough	C-Peak
	n = 565	n = 411
Dabigatran, median (range) (ng/mL)	78 (36-324)	157 (36-633)
Rivaroxaban, median (range) (ng/mL)	36.5 (17-273)	212 (17-556)
Apixaban, median (range) (ng/mL)	111.3 (22-515)	217 (45-658)
All bleeding events, n	72	52
Major bleeds, n	19	16
Intracranial	7	5
Gastrointestinal	6	6
Others	6	5
Non-major clinically relevant bleeds, n	6	—
Minor bleeds, n	47	36

Abbreviation: DOAC, direct oral anticoagulant.

treatment, another 20% during the subsequent 3 months, and the remaining between the 7th and 12th months.

The DOAC anticoagulant levels (median [min-max]) measured at C-trough (n = 565) or at C-peak (n = 411) are shown in Table 2. The total interindividual variability, expressed as overall CV%, was 64.4% for dabigatran, 86.4% for rivaroxaban, and 58% for apixaban.

Figure 2 shows the individual DOAC levels measured at C-peak distributed within the four classes of anticoagulant activity (the results obtained at C-trough are shown in Figure S1). We performed univariate and multivariate analyses for the risk of bleeding complications comparing the patients with C-peak levels in the lowest level (Class I) versus those with concentrations in the higher levels (Classes II-III-IV) and according to whether the CHA2DS2VASc or HASBLED scores indicated low or high risk. As can be seen in Table 3, only the C-peak levels in the higher classes were associated with an increased risk of bleeding at multivariate analysis (odds ratio 2.7; 95% confidence interval: 1.3 to 5.4). Detailed results of analyses for all the investigated characteristics are reported in Table S1.

When the individual DOAC levels were also analyzed in relation to the expected plasma concentrations after therapeutic doses for each drug,[19] the C-peak levels of patients with bleeding events were more prevalent among the high responders (19.5%) than in the sum of normal plus low responders (11.1%, $P = .046$), whereas no difference was detected when C-trough levels were considered (Table 4). Using normalized dosage results (Figure 3), only the C-peak median levels were higher in patients with bleeding complications than in those without bleeding complications ($p = .003$).

4 | DISCUSSION

The DOACs are currently administered at fixed doses without the need for laboratory testing and dose adjustments, except in some special clinical conditions.[20–23] However, a high interindividual variability in drug plasma levels has been shown with all DOACs and an association between plasma levels and major events had been highlighted by FDA reports on DOAC phase III clinical studies.[2–4]

As for any anticoagulant drug, major bleeding complications may also occur during treatment with DOACs, accounting for nearly 3% patient-years.[24] Consequently, efforts aimed at increasing efficacy/safety should be devoted to improving patients' health.[25]

Recently, we reported on the significant relationship between low drug levels and thrombotic complication in NVAF patients followed for 1 year after DOAC testing at steady state at the beginning of treatment.[13] The risk of thrombotic events during follow-up was higher in patients at high cardiovascular risk who had low DOAC anticoagulant levels measured at C-trough. These results support the concept that a "good anticoagulation level" is necessary for effective protection from cardiovascular complications, especially in those patients at high risk.

The present observational study was conducted on the same NVAF patient population and using the same plasma samples. The study aimed at assessing the possible relationship between the C-trough or C-peak anticoagulant levels, measured nearly at the beginning of treatment, and the occurrence of bleeding events during 1-year follow-up after blood sampling. We found a significantly higher prevalence of patients with bleeding events among those patients who had high C-peak anticoagulant levels than those with the lowest C-peak values. Furthermore, the C-peak results, normalized for the median peak result of the corresponding DOAC, were significantly higher in patients with than without bleeding complications. Finally, patients with bleeding events were more prevalent among those who can be considered high responders after therapeutic doses of each drug (as proposed by Douxfils et al)[19] than among those normal or low responders. In contrast, the C-trough results did not show any relationship with occurrence of bleeding events (Table 3, Figure S1); however, we cannot exclude that these negative results may also be imputed to the relatively low number of patients examined. Unfortunately, specific DOAC measurements at the time of bleeding events were not available. The value of C-trough and C-peak, as already observed in some patient populations treated with low-molecular-weight heparins,[2–4] could be related to thromboembolic or bleeding risk, respectively.

In this study, all the bleeding events (MB, NMCRB, MinB) have been evaluated regardless of their clinical importance. The inclusion of MinB may be questioned due to their low clinical relevance. However, they may have emotional impact and may affect the quality of life of treated patients. It may be expected that their occurrence would be influenced by the intensity of anticoagulant activity. We included in this evaluation not only spontaneous but also post-traumatic hemorrhages. Although the occurrence of the latter is associated with trauma, the intensity of anticoagulation at the time of

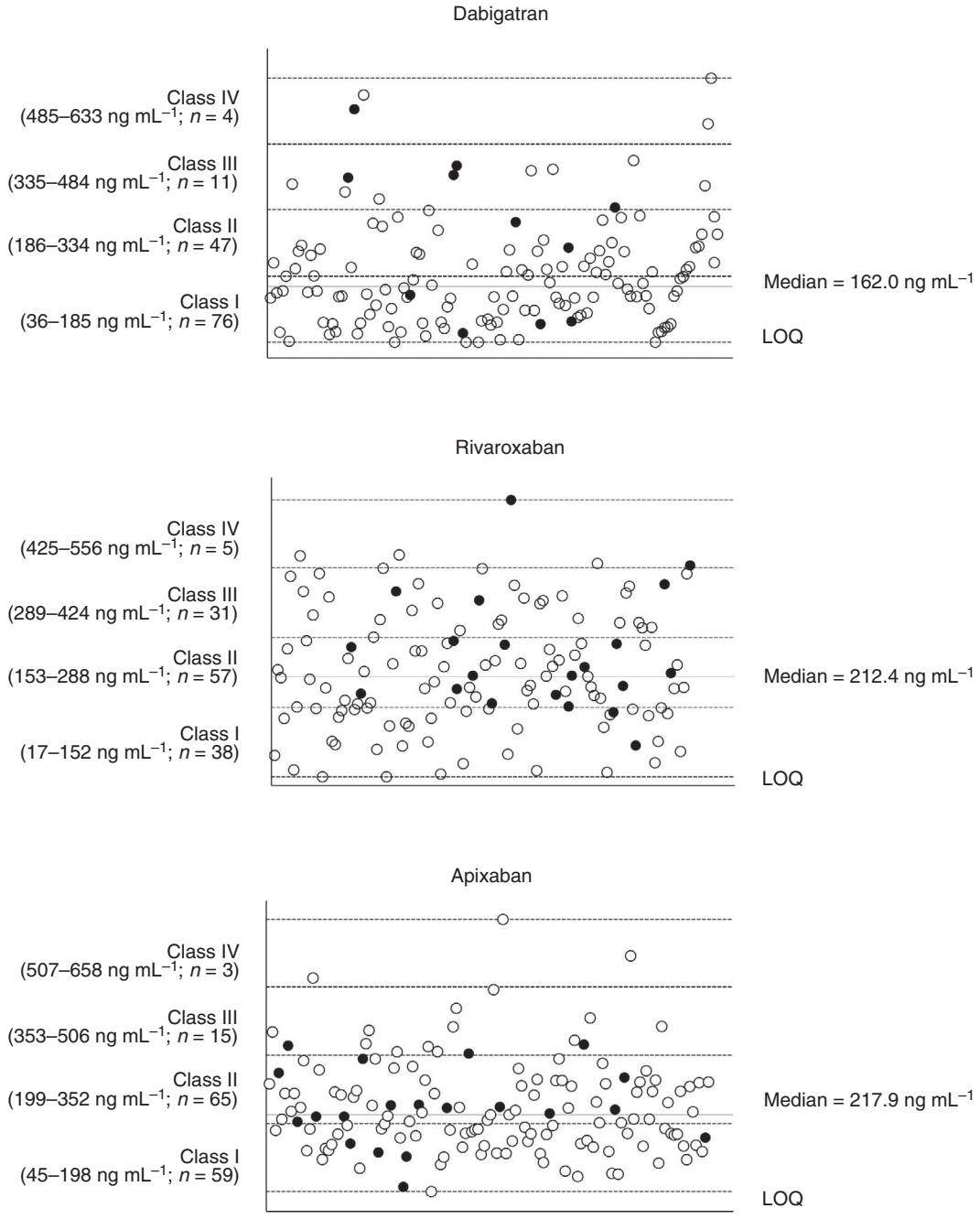

FIGURE 2 Distribution of dabigatran, rivaroxaban, or apixaban plasma levels measured at peak into the four classes of drug levels, determined from the limit of quantification (LOQ) to the highest concentration. Filled and empty circles identify patients with bleeding events or none, respectively

trauma may affect the severity and duration of bleeding. No difference was found in the C-trough or C-peak levels between patients who had spontaneous or posttraumatic hemorrhages.

It is well known that some patients may have considerable risk of bleeding that is independent of anticoagulation, owing to various conditions such as age, comorbidities, comedications, renal function, and others.[2,4] We, therefore, looked at other possible causes of differences between patients with/without bleeding complications; only C-peak levels were associated with a higher odds ratio for bleeding occurrence at multivariate analysis (odds ratio [95% confidence interval) 2.7 [1.3 to 5.4]), whereas other characteristics (e.g., CHA_2DS_2VASc or HAS-BLED scores, age, gender, and renal function) were not.

An important question arises from the analysis of the results of our two studies conducted on this NVAF patient population. On one hand, we observed a significant relationship between the lowest C-trough DOAC plasma levels and the occurrence of thrombotic complications. On the other hand, we observed a significant association of C-peak values with bleeding events. Both these findings are reasonable since they are intrinsically related to the action of

TABLE 3 Results of univariate and multivariate analyses for the risk of bleeding complications

	Patients without bleeds	Patients with bleeds	Univariate	Multivariate
			OR (95% CI)	OR (95% CI)
C-peak results were available in only 411 patients	N = 359	N = 52		
Class level I (peak plasma levels)	162 (45.1)	12 (23.1)	Rif	
Class level II-II-IV (peak plasma levels)	197 (54.9)	40 (76.9)	2.7 (1.4-5.3)	2.7 (1.3-5.4)
	N = 493	N = 72		
CHA2DS2VASc low risk (0-1)	49 (9.9)	6 (8.3)	Rif	
CHA_2DS_2VASc high risk (≥2)	443 (89.9)	66 (91.7)	1.2 (0.5-1.9)	1.6 (0.1-15.6)
HASBLED low risk (<3)	175 (49.3)	18 (42.9)	Rif	
HASBLED high risk (≥3)	180 (49.7)	24 (57.1)	1.3(0.7-2.5)	2.9 (0.9-9.1)

Note: Comparing patients with the lowest C-peak levels (Class I) with those with higher levels (Class II-IV) and comparing patients with CHA2DS2VASc or HASBLED scores at low risk versus those at high risk. Multivariate analysis was performed for high peak plasma levels and high CHA_2DS_2VASc and HASBLED scores.
Abbreviations: CI, confidence interval; OR, odds ratio.

TABLE 4 Number of patients (with bleeding events or not, n/N) with C-trough or C-peak anticoagulant DOAC levels above, within, or below (high, normal, or low responders, respectively) the plasma concentrations expected after therapeutic doses of each drug (reported by Douxfils et al[19])

	C-trough	C-peak
	n/N	n/N
High responders	8/65 (12.3)	15/77 (19.5)
Normal responders	55/421 (13.1)	27/244 (11.1)
Low responders	9/79 (11.4)	10/90 (11.1)

Note: Reported by Douxfils et al[19]
Abbreviations: DOAC, direct oral anticoagulant.

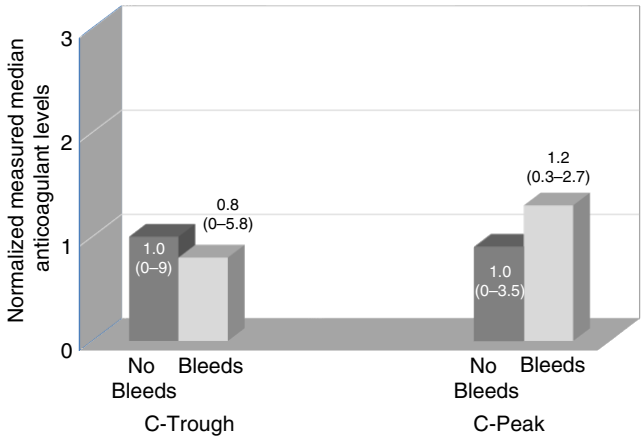

FIGURE 3 Results of normalized anticoagulant median levels assessed at C-trough and C-peak times for all DOAC drug patients with/without bleeding complications. To make the observations for different DOACs comparable, the results obtained in patients treated with different drugs were divided for the median value calculated for the corresponding molecule. DOAC, direct oral anticoagulant

any anticoagulant treatment. Anticoagulant drugs are prescribed to treat and prevent thrombotic events. While, on one hand, low drug levels may be insufficient to avoid coagulation activation and thrombus formation, on the other hand, high drug levels may favor, if not cause, bleeding complications. The best solution would be to find the optimal trade-off levels able to combat the risks of thrombosis and bleeding complications. Thanks to the many clinical studies carried out over the last decades, the best therapeutic intervals for vitamin K antagonists (VKAs) (ranging from 2.0 to 3.0 international normalized ratio [INR] for most indications) have been established, thus allowing effective protection against thrombosis, while maintaining the lowest risk of bleeding.[26] In our opinion, similar efforts for DOACs are still lacking. Altogether, the results of our two studies suggest that relatively higher DOAC levels at trough and relatively lower levels at peak would be the optimal solution to spare thrombotic and also bleeding complications. This would imply an effort to establish a more accurate therapeutic dosing to be related with the variability of treating patients.

We recognize that both the present and the previous study have important limitations. First and most importantly, the numbers of patients included in the two studies and the outcomes recorded were relatively small and insufficient to draw definitive conclusions; moreover, some C-peak results were lacking. Second, DOAC levels were measured only once at the steady state after the beginning of anticoagulant treatment and not close to the adverse events. Furthermore, changes in the drug levels during the observation period should also be taken into account, because of the intraindividual variability of drug plasma concentrations, which has been reported around 20%.[9] Finally, as observed by some observational studies,[27] adherence to treatment and its persistence during time

may be a problem for therapy with DOACs. We cannot exclude that adherence problems may have influenced our results; however, it has been shown that adherence may be improved when patients are adequately monitored,[28] as was the case in our study. For all these reasons, our studies should only be considered as preliminary observations.

In conclusion, the present study showed that the prevalence of NVAF patients with bleeding complications during treatment with DOACs was significantly higher among those who had higher C-peak anticoagulant levels. The present and the previous study, carried out on the same cohort of patients,[13] seem to indicate that a more accurate definition of an optimal therapeutic window for DOACs in NVAF patients may contribute to increase efficacy and safety of these treatments. However, we want to point out that our studies do not support any deviation in the current clinical practice, in terms of dosage or frequency of administration, from what is recommended in the product monograph of each DOAC. Owing to their limitations, these studies should only be considered as an indication for the need of larger and specifically designed clinical studies on this issue. In this regard, a much larger study, the MAS (Measure And See study; NCT03803579), is currently ongoing and is aimed at defining the relationship between anticoagulant DOAC plasma levels and adverse events in NVAF patients.

CONFLICTS OF INTEREST

S. Testa has received honoraria from Bayer Pharmaceuticals, Boehringer Ingelheim, Stago, Daiichi, BMS-Pfizer, Sobi, CSL Behring, and Roche outside the submitted work; G. Palareti has sat on advisory boards for Alfasigma, Pfizer, BMS, and Roche and has received speaker's fees from Werfen, outside the submitted work. The other authors state that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Role of each author: S. Testa: study design, first draft manuscript preparation; C. Legnani: patient identification and data analysis; E. Antonucci: data analysis; O. Paoletti: patient identification and manuscript approval; C. Dellanoce: patient identification and data analysis; B. Cosmi: manuscript revision and approval; V. Pengo manuscript revision and approval; D. Poli: manuscript revision and approval; R. Morandini acquisition of data; R. Testa acquisition of data and manuscript approval; A. Tripodi manuscript revision and approval; G. Palareti: final manuscript preparation.

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SUPPORTING INFORMATION

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

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