Increased Levels of Platelets and Endothelial-Derived Microparticles in Patients With Non-Valvular Atrial Fibrillation During Rivaroxaban Therapy

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Abstract
It is known that atrial fibrillation (AF) is associated with the procoagulant state. Several studies have reported an increase of circulating microparticles in AF, which may be linked to a hypercoagulable state, atrial thrombosis and thromboembolism. We evaluated in our study alterations in both platelet (PMP, CD42b) and endothelial-derived (EMP, CD144) microparticle levels on anticoagulant therapy with rivaroxaban in nonvalvular AF. After administration of rivaroxaban, PMP levels were increased (median, [IQR] 35.7 [28.8-47.3] vs. 48.4 [30.9-82.8] cells/μL; P = 0.012), along with an increase in EMP levels (14.6 [10.0-18.6] vs. 18.3 [12.9-37.1] cells/μL, P < 0.001). In the multivariable regression analysis, the independent predictor of post-dose change in PMPs was statin therapy (HR/C0 0.43; 95% CI 0.75, 0.10, P = 0.011). The post-dose change in EMPs was also predicted by statin therapy (HR 0.34; 95% CI 0.69, 0.01, P = 0.046). This study showed an increase in both EMPs and PMPs at the peak plasma concentration of rivaroxaban. Statins have promising potential in the prevention of rivaroxaban-related PMP and EMP release. The pro-thrombotic role of PMPs and EMPs during rivaroxaban therapy requires further study.

Keywords
platelet-derived microparticles, CD42b, endothelial-derived microparticles, CD144, nonvalvular atrial fibrillation, rivaroxaban

Introduction
Circulating microparticles (MPs) are membrane-derived structures shed into circulation from all morphotic blood elements as well as the vascular endothelium in response to cellular apoptosis or activation.1,2 Previous studies have suggested that MPs play a functional role in cardiovascular disease. Increased levels of MPs have been found in the presence of acute coronary syndromes,3 in coronary angioplasty patients4 and in patients with a history of stroke.5 Platelet-derived MPs (PMPs) were an independent risk factor for the infarct volume in patients with acute ischemic stroke.6 High levels of endothelial-derived MPs (EMPs) have been reported to impact the severity, lesion volume and outcome of acute ischemic stroke.7 Several studies have reported an increase of MPs in AF, which may be linked to a hypercoagulable state, atrial thrombosis and thromboembolism.8-13 Siwaponanan et al13 showed an increase in PMP and EMP levels in AF patients, which may reflect endothelial damage and platelet activation. In the recent study, the administration of dabigatran was associated with an increase in PMP levels in the AF patients, and the release of PMPs strongly correlated with the concentration of drug in the blood, suggesting that lower dabigatran...
concentrations were able to diminish post-drug platelet activation. However, to the best of our knowledge, no study has assessed the levels of PMPs and EMPs in patients with non-valvular AF treated with rivaroxaban depending on the concentration of anticoagulant in the blood yet. Therefore, we aimed to evaluate the levels of the MPs expressing platelet glycoprotein Ib (CD42b, PMPs) and vascular endothelial cadherin (VE-cadherin, CD144, EMPs) in patients with non-valvular AF while on rivaroxaban.

**Methods**

Blood samples from 34 patients who were screened in our department between August 2015 to August 2017 were examined. Written informed consent was obtained from each subject. The data were anonymized prior to statistical analysis. In accordance with the Declaration of Helsinki, the study was approved by the Jagiellonian University Ethics Committee (No. 122.6120.97.2015, Krakow, Poland).

**Recruitment Criteria**

The study included patients with non-valvular AF (paroxysmal, persistent, or permanent) receiving rivaroxaban anticoagulation therapy at least 7 days prior to measurement. Patients with unstable coronary artery disease (CAD), exacerbation of chronic heart failure, uncontrolled hypertension, recent (<3 months) thromboembolic events such as stroke/TIA or systemic embolism, neoplastic disease, connective tissue disease, uncontrolled thyroid disease and chronic kidney disease (CKD) stage 4 or higher, with an active infection or undergoing treatment with steroids were excluded from the study.

**Methods**

According to the protocol of the study, we determined the level of PMPs and EMPs twice, at the time of the expected minimum (22 to 24 h after intake of rivaroxaban) and maximum concentrations in the blood (1 to 3 hours after drug ingestion). Following that, we determined the peak rivaroxaban plasma concentration.

Patients received a real-life dose of rivaroxaban according to current guidelines. A standardized questionnaire was used to collect the patients’ demographics data, as well as information about their cardiovascular risk factors and current treatment. The CHA2DS2-VASc score was used to evaluate their risk of stroke or systemic embolism. We estimated the patients’ bleeding risk using the HAS-BLED score. Patients were monitored for 12 months for cases of stroke, systemic embolism and major bleeding.

**Measurement of Platelet-Derived and Endothelial-Derived Microparticle Levels**

Sixty-eight venous blood samples were collected into CTAD vacuum glass tubes (Vacutainer™, Becton Dickinson). Platelet-free plasma samples were prepared following recommendations by the International Society on Thrombosis and Haemostasis. PMP and EMP levels were quantified through the direct fluorescence method using a flow cytometry assay (FACSCalibur flow cytometer, Becton Dickinson, USA), as described previously. Values were reported as counts/μL of platelet-poor plasma. The change in MP levels (ΔPMPsand ΔEMPs, respectively) was defined as the difference between the number of MPs at times of expected peak and trough levels of rivaroxaban. The technicians performing these measurements were blinded to the treatment groups.

**Measurement of Rivaroxaban Concentration**

Venous blood was drawn into tubes containing Monovette vacuum tubes (S-Monovette, Sarstedt), each containing 3.2% trisodium citrate solution (10:1). Plasma concentrations of rivaroxaban were determined in 24 patients with the BCS XP Automated Blood Coagulation Analyzer System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), using a DiXal assay (Hyphen BioMed, Neuville-sur-Oise, France) as per the manufacturer’s instructions.

**Statistical Analysis**

The power of the study was 90% to detect a 10% difference in median MPs, based on a previous study. Thirteen patients were required in each group in order to demonstrate at least the minimum difference in MP concentrations. Unless otherwise specified, variables were reported as the mean ± standard deviation or the median and interquartile range (IQR). The quantitative variables were tested for normality of distribution with the Shapiro-Wilk test and compared between the groups with the t-Student test or the Mann-Whitney U test. Pre- and post-rivaroxaban MP levels were checked with the Wilcoxon sign test. The linear relationship between continuous variables was evaluated with Pearson’s correlation test or Spearman’s rank test. The clinical and laboratory variables that showed the association with MPs in the univariate model (P < 0.10) were included in the multivariate analysis. The models were adjusted for age. P-values < 0.05 were considered statistically significant and data were analyzed using Statsoft Inc STATISTICA 13.0 software.

**Results**

**Clinical Characteristics of the Study Subjects**

The clinical characteristics are summarized in Table 1. The study group was comprised of 34 patients with AF (paroxysmal n = 12 [35.3%]; persistent n = 10 [29.4%]; permanent n = 12 [35.3%]) treated with rivaroxaban. This includes 23 patients (67.7%) taking a standard dose of 20 mg once daily (o.d.) and 11 patients (32.3%) receiving a reduced dose of 15 mg o.d. Almost all patients (n = 33; 97.1%) were at a high risk for stroke (CHA2DS2-VASc score ≥2). A quarter of patients (n = 9; 26.5%) had a high risk of bleeding (HAS-BLED score.
Patients who were treated with 20 mg of rivaroxaban were more frequently males, were younger, had a lower prevalence of peripheral artery disease and CKD grade 3, were undergoing antiplatelet therapy, with lower CHA2DS2-VASc and HAS-BLED scores, and had both higher creatinine clearance and hemoglobin compared to those treated with a dose of 15 mg o.d. PMP and EMP levels before and after rivaroxaban administration were not associated with the concentration of rivaroxaban (Table 1).

**Plasma concentrations of PMPs and their determinants.** PMP levels after anticoagulant administration were higher in patients older than 65 years and treated with angiotensin II receptor blockers. Patients with hypercholesterolemia treated with a statin had lower post-rivaroxaban PMPs (Supplemental Table 1). There was no difference in PMPs before anticoagulant administration in the aforementioned groups.

PMP levels increased after rivaroxaban administration (median [IQR] 35.7 [28.8-47.3] vs. 48.4 [30.9-82.8] cells/μL; P = 0.012) (Figure 1). In the multivariable regression analysis, statin therapy was the only independent predictor of ΔPMPs (hazard ratio [HR] 0.43; 95% confidence interval [CI] -0.75,-0.10; R2 = 0.18; Table 2).

**Plasma concentrations of EMPs and their determinants.** EMP levels before taking rivaroxaban correlated positively with age (r = 0.35, P = 0.043), the CHA2DS2-VASc score (r = 0.36, P = 0.036), and the HAS-BLED score (r = 0.37, P = 0.029). Patients older than 65 years had higher EMP levels before anticoagulant administration and a trend toward higher EMPs after anticoagulant administration (Supplemental Table 1). Patients treated with a statin had a lower EMPs count both before and after rivaroxaban administration. Patients with hypercholesterolemia had lower EMP levels after anticoagulant administration and a trend toward lower EMP levels before anticoagulant administration.

EMP levels increased after taking anticoagulants (14.6 [10.0-18.6] vs. 18.3 [12.9-37.1] cells/μL, P < 0.001) (Figure 1).
The independent predictor of ΔEMPs was statin therapy (HR = 0.34; CI = 0.01, 0.69; R2 = 0.01; Table 3).

12-Month Follow-Up. Neither ischemic stroke nor systemic thromboembolism were observed during the 12-month follow-up period. A new thrombus in the left ventricular apex was revealed with echocardiography in 1 patient treated with a reduced dose of rivaroxaban. No major bleeding was observed during the follow-up. Minor nasal bleeding was observed in 1 patient receiving a standard dose of rivaroxaban.

Discussion

To the best of our knowledge, this is the first study of AF patients to demonstrate flow cytometric analysis of PMP and EMP concentrations in nonvalvular AF patients on anticoagulant therapy with rivaroxaban depending on the expected minimum and maximum concentration of anticoagulant in the blood. Unexpectedly, we observed that the administration of rivaroxaban was associated with an increase in PMP and EMP levels, suggesting post-drug endothelial and platelet activation. Statin therapy was associated with a lower post-rivaroxaban increase in PMPs and EMPs.

PMPs and EMPs are an essential part of the physiological clotting process and of thrombosis. In vitro studies have shown that PMPs released from activated platelets may be involved in blood clot formation and fibrinolysis. In our study, we used PMPs expressing platelet glycoprotein Ib (GPIb, CD42b), a marker of platelet activation. It is a component of the GPIb-V-IX complex on platelets that binds von Willebrand factor and mediates platelet plug formation and adhesion to the subendothelium at sites of injury. We assessed in our study

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**Figure 1.** Panel A, B. Associations between administration of rivaroxaban (pre, post) and PMP, EMP levels in patients with AF. Panel C, D. Post-rivaroxaban change in PMPs level (ΔPMPs) and EMPs level (ΔEMPs) according to statin use. Values are presented as a median and interquartile range, and black points indicate outliers.

**Table 2.** Multivariable Regression Analysis of ΔPMPs (R2 = 0.18).a

| Variable | Univariate analysis | Multivariate analysis |
|----------|-------------------|---------------------|
|          | HR (95% CI) | P-value | HR (95% CI) | P-value |
| Age (years) | 0.34 (0.01, 0.67) | 0.052 | — | — |
| Statin | −0.43 (−0.75, −0.10) | 0.012 | −0.43 (−0.75, −0.10) | 0.011 |
| ARB | 0.29 (0.06, 0.63) | 0.102 | — | — |

For abbreviations, see Table 1.

**Table 3.** Multivariable Regression Analysis of ΔEMPs (R2 = 0.12).a

| Variable | Univariate analysis | Multivariate analysis |
|----------|-------------------|---------------------|
|          | HR (95% CI) | P-value | HR (95% CI) | P-value |
| Age (years) | 0.33 (0.01, 0.67) | 0.059 | — | — |
| HAS-BLED score | 0.32 (0.01, 0.67) | 0.061 | — | — |
| Statin | −0.35 (−0.69, −0.01) | 0.046 | −0.34 (−0.69, −0.01) | 0.046 |

For abbreviations, see Table 1.
EMP{s} expressing vascular endothelial cadherin (VE-cadherin, CD144), a marker of endothelial activation, which is an endothelial-specific adhesion protein located at the junctions between endothelial cells and plays a crucial role in endothelial barrier function and angiogenesis.

There are limited data on MPs level during anticoagulation. Siwaponanan et al. demonstrated a marked increase in total circulating MP levels and evidence of platelet activation and endothelial damage, as demonstrated by increased PMP and EMP levels, in the AF patients treated predominantly with warfarin. Duarte et al. reported higher PMP in the AF patients taking rivaroxaban and warfarin compared to the non-AF group. The authors suggested that warfarin or rivaroxaban use decreased thrombin generation triggering, thereby attenuating the thrombotic potential of MPs. In both studies, the relationship between the time of blood collection and administration of an anticoagulant was not evaluated. In our study, we collected blood for analyses twice, at time intervals for the predictable minimum and maximum rivaroxaban blood levels, because our goal was to evaluate the expression of PMP and EMP depending on the expected concentration of anticoagulant in the blood.

Rivaroxaban, a direct activated factor X inhibitor (FXa), causes both delayed thrombin generation and the formation. It targets free and clot-bound FXa and FXa in the prothrombinase complex, which activates prothrombin to thrombin. Looking at the mechanism of action of rivaroxaban, we expected that after drug administration, levels of PMPs and EMPs would decrease during the period of its predicted highest anticoagulant blood concentration. Unexpectedly, we observed that the administration of rivaroxaban was associated with an increase in PMP and EMP levels which may suggest a prothrombotic effect. Regarding baseline MPs levels, the expression of EMPs was higher before drug administration in patients >65 years of age. Therefore, the expression of certain MPs may be related to the drug’s peak action and influenced by age. We hypothesize that feedback between FXa thrombin generation, PMPs and EMPs may explain alterations in MP levels observed in our study. However, the exact mechanism and clinical significance of this remains to be established.

MP{s} have primarily been described as having procoagulant properties. MP{s} express anionic phospholipids such as phosphatidylserine on their outer leaflet, which assemble calcium-dependent coagulation factors on the surface of MPs, thus forming tenase and/or prothrombinase complexes, leading to thrombin formation. Moreover, tissue factor expressed on endothelial cells and plays a crucial role in endothelial barrier function and angiogenesis.

In the AF patients anticoagulated with rivaroxaban, we hypothesize that feedback between FXa thrombin generation, PMPs and EMPs may explain alterations in MP levels observed in our study. However, the exact mechanism and clinical significance of this remains to be established.

Previous studies have shown vasoprotective effects of rivaroxaban. Alvarez et al. showed in their study on human umbilical vein endothelial cells that rivaroxaban enhanced viability, growth, and migration of endothelial cells, suggesting its protective effects on the endothelium. Also, a study by Pham et al. showed that rivaroxaban attenuated the development of endothelial dysfunction in diabetic mice. The complexity of the mechanism of rivaroxaban might have contributed to the discrepancies in the results of studies on MPs function, as it has been shown to increase endothelial nitric oxide (NO) synthase expressions in endothelial progenitor cells and inhibit protease-activated receptors signaling in atrial fibroblasts.

In the AF patients anticoagulated with rivaroxaban, we observed that treatment with statins was associated with a lower increase in ΔPMPs and AEMP{s}. It should be emphasized that statin-treated patients at baseline had a lower PMPs and EMPs count prior to the administration of rivaroxaban (Supplemental Table 1). This may be related to the complex effect of statins, as they inhibit platelet activation and reduce
endothelial damage regardless of rivaroxaban. The anticoagulant properties of statins are related to decreased tissue factor expression, reduced thrombin generation and attenuation of thrombin-catalyzed reactions, as well as increased endothelial thrombomodulin expression.

Our observations may help in developing individual treatment strategies, particularly in patients with thrombosis despite the use of NOAC. A protective effect of statin therapy in the rivaroxaban anticoagulated patients may be beneficial in the AF patients with coexisting hypercholesterolemia, although further studies are required.

Study Limitations

Despite careful selection of the study group, the influence of comorbidities on PMP and EMP in this subgroup of patients could not be ruled out. We cannot ignore that the size of the investigated group was limited. However, the number of subjects was sufficient to detect differences between groups given the results of the power calculation. While the number of patients taking statins allowed for the evaluation of the correlation with the blunt increase in microparticles, two of the other potential factors, antiplatelets and ARBs had much lower numbers and likely underpowered to be able to assess. The limitation of our study may also be the lack of differentiation between the types of statins that were used by the patients, as well as their dosages. Differences in the pharmacokinetics of statins could influence the results observed. Due to the high risk of thromboembolism in the study group, we did not include patients with AF and without anticoagulation treatment.

Results of our study have provided useful information regarding the trends of PMPs and EMPs expression; however, it did not give us data on whether it is a persistent or only temporary phenomenon and whether it is a result of direct activation of platelets and endothelium or a drug effect. Indeed, taking more serial measurements during the period of rivaroxaban reaching its peak concentration could shed more light, and this requires further research. Probably the ongoing studies to no fours study may falsify the lack of differentiation potential factors, antiplatelets and ARBs had much lower numbers and likely underpowered to be able to assess. The limitation of our study may also be the lack of differentiation between the types of statins that were used by the patients, as well as their dosages. Differences in the pharmacokinetics of statins could influence the results observed. Due to the high risk of thromboembolism in the study group, we did not include patients with AF and without anticoagulation treatment.

Conclusion

In patients with non-valvular AF, rivaroxaban administration is associated with an increase in PMP and EMP levels. The release of microparticles is influenced by the statin; it causes a smaller increase after taking the drug, which suggests a protective, anticoagulant effect. This mechanism may enhance anticoagulation therapy in patients with atrial fibrillation and reduce the risk of thromboembolism. Our findings provided are scientifically relevant and warrant further studies with a larger cohort. Due to the above-mentioned limitations of the study, we are not entitled to stop using anticoagulant therapy.

Acknowledgments

The authors gratefully acknowledge Prof. Ewa Wypasek from the Laboratory of Molecular Biology in John Paul II Hospital and both Prof. Piotr Marek Radziwon and Mateusz Dziermiąńczuk from the Regional Center for Blood Donation and Blood Treatment in Bialystok for their support in determining microparticles using a flow cytometer.

Author Contributions

The concept and design of the study: ALM, MO, data collection: ALM, MKW, LTP data analysis: ALM, LD, MKW, LTP, PP, MO, draft manuscript preparation: ALM, LD, MO, final manuscript approval: ALM, LD, MKW, LTP, PP, MO.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was supported by a grant from Jagiellonian University Medical College (no K/ZDS/005459) and by the science fund of the John Paul II Hospital, Cracow, Poland (no. FN9/2021 to ALM).

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Supplemental Material

Supplemental material for this article is available online.

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