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Prognostic implications and procoagulant activity of phosphatidylserine exposure of blood cells and microparticles in patients with atrial fibrillation treated with pulmonary vein isolation

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Abstract. The present study aimed to evaluate the procoagulant effects of phosphatidylserine (PS) exposure on blood cells and microparticles (MPs), and examine its role in predicting early recurrence atrial fibrillation (ERAF) in patients with atrial fibrillation (AF) treated with pulmonary vein isolation (PVI). Blood samples were obtained from 40 healthy controls and 56 patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 days and 7 days following PVI. The exposure of PS (PS⁺) to blood cells (platelets, erythrocytes and leukocytes) and MPs was detected using flow cytometry. The procoagulant activity was evaluated by coagulation time, and the formation of factor Xa (FXa) and thrombin. In addition, independent factors associated with PS⁺ blood cells and MPs, and significant predictors of ERAF following PVI were investigated by statistical analyses. The numbers of PS⁺ blood cells and MPs were significantly increased by PVI (P<0.01). A significant decrease in coagulation time, and increases in FXa and thrombin were exhibited in the PS⁺ blood cells and MPs from patients with AF treated with PVI, whereas these alterations were inhibited by either lactadherin or anti-tissue factor (P<0.01). The maximum power of the PVI was significantly associated with

platelet-derived MPs, and high-sensitivity C-reactive protein (hs-CRP) was closely associated with leukocyte-derived MPs and endothelial-derived MPs (EMPs) (P<0.01). In addition, hs-CRP and EMPs >355/ μ l were identified as independent predictors of ERAF (P<0.05). The increased numbers of PS⁺ platelets, erythrocytes, leukocytes and MPs contributed to the procoagulant state of AF, and hs-CRP and EMPs were able to predict ERAF following PVI.

Introduction

Atrial fibrillation (AF) is a common and serious type of arrhythmia, which is characterized by an irregular and rapid heartbeat (1). Clinically, pulmonary vein isolation (PVI) is a feasible, safe and effective treatment method for patients with AF; however, it is associated with the potential risk of peri-procedural stroke, despite appropriate anticoagulation (2). As a high risk factor in the treatment of AF, thrombosis can seriously affect the effects of PVI treatment on AF, and appropriated anticoagulation is always used to reduce the risk of thrombosis (3,4). It has been reported that P-selection⁺ platelets, platelet-derived microparticles (PMPs) and leukocyte-derived MPs (LMPs) are increased immediately following PVI and persist in the subsequent 24 h, which can significantly increase the risk of thrombosis (5-7). In terms of the predominant underlying mechanisms, increased thromboembolic risk during PVI may be explained by endocardium denaturation and hemodynamic alterations due to energy applied in catheterization and electrical cardioversion (8-10). However, associated mechanisms underlying how procoagulant activity (PCA) is affected by PVI require further investigation.

As an anionic lipid, phosphatidylserine (PS) is an important phospholipid membrane component located between the inner and cytoplasmic leaflet of the bilayer (11). Exposure of PS on the outer leaflet and microparticles (MPs) are found in the process of apoptosis (12,13). In addition, PS exposure on the outer membrane surface can function as a docking site for various coagulation proteins, including factor (F)VII, FIX,

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FV, FVIII, FX and prothrombin, and promote the formation of thrombin (14,15). As a primary cellular initiator of blood coagulation via interaction with coagulation FVII, tissue factor (TF) is generally quiescent unless it resides on a cell membrane containing PS (16). It has been reported that TF is involved in the generation of MPs, cell-associated PCA in diabetes mellitus and disseminated intravascular coagulation associated with severe infections (17,18). Therefore, PS and TF are considered to contribute to PCA and outcomes for patients with AF following PVI. However, whether and how PS exposure of blood cells and MPs contributes to PCA following PVI requires further investigation.

The post-ablation recurrence of AF remains a major clinical problem, occurring in 20-60% of patients during follow-up (19,20). In addition, there is a paucity of data on the correlations of early recurrence AF (ERAF) following PVI with PS⁺ blood cells and MPs. In the present study, the exposure of PS on blood cells (platelets, erythrocytes and leukocytes) and MPs were detected in patients with AF treated with PVI, and the PCA was evaluated by determining coagulation time, and the formation of FXa and thrombin. Subsequently, independent factors associated with PS⁺ blood cells and MPs, and the predictors of ERAF following PVI were investigated. The findings may reveal the mechanisms underlying the promoting effects of PS⁺ blood cells and MPs on PCA following PVI, and its clinical prognostic value on ERAF.

Materials and methods

Subjects. A total of 56 patients with AF, including 48 patients with paroxysmal AF and 8 with non-paroxysmal AF, who had undergone a transseptal PVI procedure were selected from the Second Hospital of Harbin Medical University (Harbin, China) between November 2013 and April 2015. AF was identified according to the Heart Rhythm Society expert consensus statement (21). An additional 40 healthy subjects were recruited from the preoperative clinic, which were included as the control group. Patients with a history of previous myocardial infarction, surgery or ablation procedure within 3 months, congenital heart disease, history of connective tissue diseases or chronic inflammatory conditions, acute/chronic infection or chronic renal/liver failure were excluded from the study. The study was approved by the local ethics committee of the Second Hospital of Harbin Medical University and performed with written informed consent from the patients.

PVI. The PVI was performed on patients with AF, as previously described (9). In brief, warfarin was first titrated to an international normalized ratio of 1.8-2.5 2 days prior to PVI by oral anticoagulation. Trans-septal puncture was guided by transesophageal echocardiography to exclude thrombi in the left atrium. Subsequently, 50 IU/kg unfractionated heparin was administered to maintain an activated clotting time between 300 and 350 sec. Ablation of the pulmonary veins was performed with a delivered power of 30-35 W and irrigation rates of 17-30 ml/min. All patients were scheduled for 24 h Holter recording at baseline (prior to PVI), and at follow-up at 1, 3 and 6 months. ERAF was defined as AF

occurring at any time beyond the 3-month blanking period following ablation.

Flow cytometric analysis of PS-exposed blood cells. Peripheral vein blood samples were collected from the patients with AF prior to PVI (baseline), at the end of transseptal puncture (0 h), 1 h post-PVI, and 1, 3 and 7 days post-PVI. Platelets (300 x g for 15 min at 20°C), erythrocytes (1,500 x g for 10 min at room temperature) and leukocytes (300 x g for 5 min at room temperature) were isolated by centrifugation and Percoll density gradients (22,23). These blood cells, at a density of 1x10⁶, were adjusted to a final volume of 45 µl in Tyrode's buffer, and then incubated with 4 nM Alex Fluor 488-lactadherin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 15 min at room temperature in the dark (diluted with 150 µl Tyrode's buffer). Finally, the exposure of PS on the blood cells was detected using flow cytometry (FACSAria, BD Biosciences, Franklin Lakes, NJ, USA).

Flow cytometric analysis of PS-exposed MPs. The MPs were isolated from platelet-free plasma of peripheral vein blood samples by centrifugation (350 x g for 15 min at 4°C) and Tyrode's buffer (10). The phenotype of the MPs was identified, as previously described (22), and MPs were bound by lactadherin (Alex Fluor 488-lactadherin) to evaluate the exposure of PS. In detail, platelet-derived MPs (PMPs), endothelial-derived MPs (EMPs), leukocyte-derived MPs (LMPs), erythrocyte-derived MPs (RMPs) and tissue factor-MPs (TF-MPs) were identified by lactadherin⁺ CD41a⁺, CD31⁺/CD41a⁻, CD45⁺, CD235a⁺ and CD142, respectively (14,24). The numbers of various MPs were calculated using a Trucount Tube (BD Biosciences) with a precise number of fluorescent beads (48,678; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) (22).

Subcellular localization of PS by microscopy. The subcellular localizations of PS on platelets, leukocytes and erythrocytes were observed via microscopic fluorescence observation. Briefly, 50 µl suspensions of platelets, leukocytes and erythrocytes at a density of 1x10⁶ were collected at baseline and 1 day post-PVI. These cells were then incubated with 128 nM Alex Fluor 488-lactadherin and propidium iodide (PI) for 10 min at room temperature in the dark. Following removal of the unbound dye by PBS containing 0.02% Triton X-100 (v/v), images were captured using an LSM 510 Meta confocal microscope (Carl Zeiss AG, Jena, Germany) (22).

PCA assays. The PCAs of the platelets, leukocytes, erythrocytes and MPs at baseline, and 0, 1 h, 1 day, 3 days and 7 days post-PVI were measured in 100 µl citrate plasma using a single-stage recalcification time assay with an Amelung KC4A-coagulometer (Labcon, Heppenheim, Germany) as previously described (14). The effects of 128 nM lactadherin and 40 µg/ml anti-TF on the PCAs of the platelets, erythrocytes and MPs (1 day post-ablation) and leukocytes (3 day post-ablation) were also analyzed (22).

FXa and thrombin formation assays. FXa and thrombin formation assays were performed, as previously described (22). A universal microplate spectrophotometer (PowerWave XS;

Bio-Tek, Instruments, Inc., Winooski, VT, USA) was used to detect the quantities of FXa and thrombin at 405 nm. The effects of lactadherin (128 nM) and anti-TF (40 $\mu\text{g/ml}$) on blood cells and MPs were also evaluated.

Statistical analyses. Continuous variables were examined for normal distribution using a Shapiro-Wilk test. Normally distributed variables are expressed as the mean \pm standard deviation and significance was analyzed using Student's t-test or repeated-measures analysis of variance. Non-normally distributed variables are expressed as the median with interquartile range and significance was analyzed using a Mann-Whitney U-test. Categorical variables are expressed as numbers (frequency) and were compared using a χ^2 test or Fischer's exact test. Stepwise multivariate linear regression analyses were used to assess independent factors associated with PS⁺ blood cell and MPs. Receiver operating characteristic curve analysis (ROC) was used to determine the optimum cut-off levels of PS⁺ blood cells and MPs in the prediction of ERAF following PVI. Univariate and multivariate Cox proportional hazard analyses were performed to investigate the predictors of ERAF post-PVI. Among the above analytical methods, $P < 0.05$ was considered to indicate a statistically different difference. All statistical analyses were performed by SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Clinical characteristics of patients with AF who underwent PVI. The clinical characteristics of the patients with AF who underwent PVI were evaluated. As shown in Table I, no significant differences were found in age, male/female, body mass index, diabetes mellitus, coronary artery disease, hypertension, previous stroke/transient ischemic attack, smoking, dyslipidemia, leukocyte counts, prothrombin time or D-dimer in the patients with AF, compared with the controls. However, significantly shorter activated partial thromboplastin time and higher fibrinogen were found in the patients with AF, compared with the control group. Among these patients, the levels of high-sensitivity C-reactive protein (Hs-CRP), CHA₂DS₂-VASc score, left ventricular ejection fraction (LVEF) and left atrial diameter (LAD) were 2.5 (1.3-5.3) mg/l, 1.2 \pm 0.3, 61.18 \pm 10.22% and 34.97 \pm 5.84 mm (23.21% >40 mm), respectively. In addition, between one and three types of antiarrhythmic medications were used by these patients, including angiotensin converting enzyme inhibitor/angiotensin receptor blockers (53.57%) and statins (35.71%).

In the treatment of AF, segmental PVI (5.38%), circumferential pulmonary vein ablation (48.39%), left atrium linear ablation (16.13%), right atrium linear ablation (20.43%) and electrogram-based ablation (9.68%) were performed. During these procedures, the total ablation time was 86.20 \pm 39.01 min and the maximum power was 28.82 \pm 6.71 W. Following ablation, the curative rate was 74.73% and ERAF was detected in 42.65% patients at the 3 month follow-up. Late recurrence of AF occurred in 15.27% patients, whereas 62.5% patients were confirmed to have delayed curing at subsequent follow-up.

Table I. Clinical characteristics of patients with AF treated with pulmonary veins isolation.

Characteristic	Control (n=40)	AF (n=56)
Age (years)	54.23 \pm 7.10	55.25 \pm 7.70
Male/female	25/15	34/22
BMI (kg/m ²)	23.43 \pm 3.23	23.75 \pm 3.82
DM, n (%)	4 (10%)	6 (10.71%)
CAD, n (%)	3 (7.5%)	5 (8.92%)
Hypertension, n (%)	12 (30%)	19 (33.92%)
Previous stroke/TIA, n (%)	3 (7.5%)	4 (7.14%)
Smoking, n (%)	4 (10%)	7 (12.5%)
Dyslipidemia, n (%)	3 (7.5%)	4 (7.14%)
Leukocyte counts (10 ³ / μl)	6.74 \pm 1.35	6.91 \pm 1.62
PT (sec)	11.71 \pm 1.02	11.62 \pm 0.93
APTT (sec)	44.23 \pm 9.15	42.61 \pm 7.82 ^a
Fibrinogen (g/l)	2.73 [1.73-4.24]	3.43 [2.13-5.31] ^a
D-dimer (ng/ml)	98 [45-172]	110 [32-145]

Data are presented as the mean \pm standard deviation, number (%) and mean [range]. ^a $P < 0.05$, compared with the control. AF, atrial fibrillation; BMI, body mass index; DM, diabetes mellitus; CAD, coronary artery disease; TIA, transient ischemic attack; PT, prothrombin time; APTT, activated partial thromboplastin time.

PS exposure of blood cells. The platelets, leukocytes and erythrocytes exposed to PS were detected by lactadherin binding using flow cytometry. At baseline, PS⁺ platelets, leukocytes and erythrocytes, and TF⁺ leukocytes were significantly higher in patients with AF, compared with those in the control ($P < 0.01$), whereas no significant differences were revealed in the patients with paroxysmal and non-paroxysmal AF (data not shown). Following treatment with PVI, the number of PS⁺ platelets increased with time until a peak at 1 day (17.53 \pm 5.23 \times 10⁹/l; $P < 0.01$), as shown in Fig. 1A. PS⁺ erythrocytes and leukocytes were increased with a peak at 1 h (26.23 \pm 5.31 \times 10⁹/l; $P < 0.001$) and 3 days (6.63 \pm 2.90 \times 10⁸/l; $P < 0.01$), respectively (Fig. 1B and C). TF⁺ leukocytes were also increased with time, which peaked at 1 h (7.23 \pm 3.20 \times 10⁸/l; $P < 0.01$), as shown in Fig. 1D.

To further identify the presence of PS on platelets, leukocytes and erythrocytes in the patients with AF following PVI, the subcellular localizations of PS on these cells were observed. No fluorescence was observed on the membranes of platelets, leukocytes or erythrocytes in the patients with AF at baseline, whereas fluorescence was observed in the platelets, leukocytes and erythrocytes from the patients with AF at 1 day post-PVI treatment (Fig. 1E-H).

Numbers of MPs. The effects of PVI on the numbers of MPs were evaluated. As shown in Table II, the PS⁺ MPs predominantly originated from platelets, leukocytes and endothelial cells. The numbers of total MPs, PMPs, LMPs, EMPs and TF-MPs were all significantly higher in the patients with AF, compared with those in the control ($P < 0.01$), whereas no

Table II. Numbers of PS⁺ MPs in patients with AF treated with pulmonary veins isolation.

PS ⁺ (μl)	Control (n=40)	Patients with AF (n=56)					
		Baseline	0 h	1 h	1 day	3 days	7 days
Total MPs	1,560±143	1,800±276 ^b	1,999±289 ^a	2,399±407 ^a	3,201±552 ^a	2,998±473 ^a	2,300±398 ^a
PMPs	835±170	1,143±245 ^b	1,276±247 ^a	1,528±279 ^a	2,023±376 ^a	1,846±287 ^a	1,485±268 ^a
LMPs	205±43	266±52 ^b	283±6 ^a	514±124 ^a	671±189 ^a	488±101 ^a	411±98 ^a
EMPs	136±23	174±34 ^b	226±56 ^a	302±74 ^a	367±99 ^a	340±86 ^a	277±67 ^a
RMPs	29±19	31±19	45±25 ^a	54±33 ^a	72±37 ^a	67±35	60±29 ^a
TF-MPs	33±12	43±13 ^b	54±14 ^a	68±22 ^a	84±32 ^a	104±78 ^a	66±27 ^a

Data are presented as the mean ± standard deviation. ^aP<0.01, compared with different time points; ^bP<0.01, compared with the control. PS, phosphatidylserine; MPs, microparticles; AF, atrial fibrillation; PMPs, platelets-MPs; LMPs, leukocyte-MPs; EMPs, endothelial-MPs; RMPs, erythrocyte-MPs; TF-MPs, tissue factor-MPs.

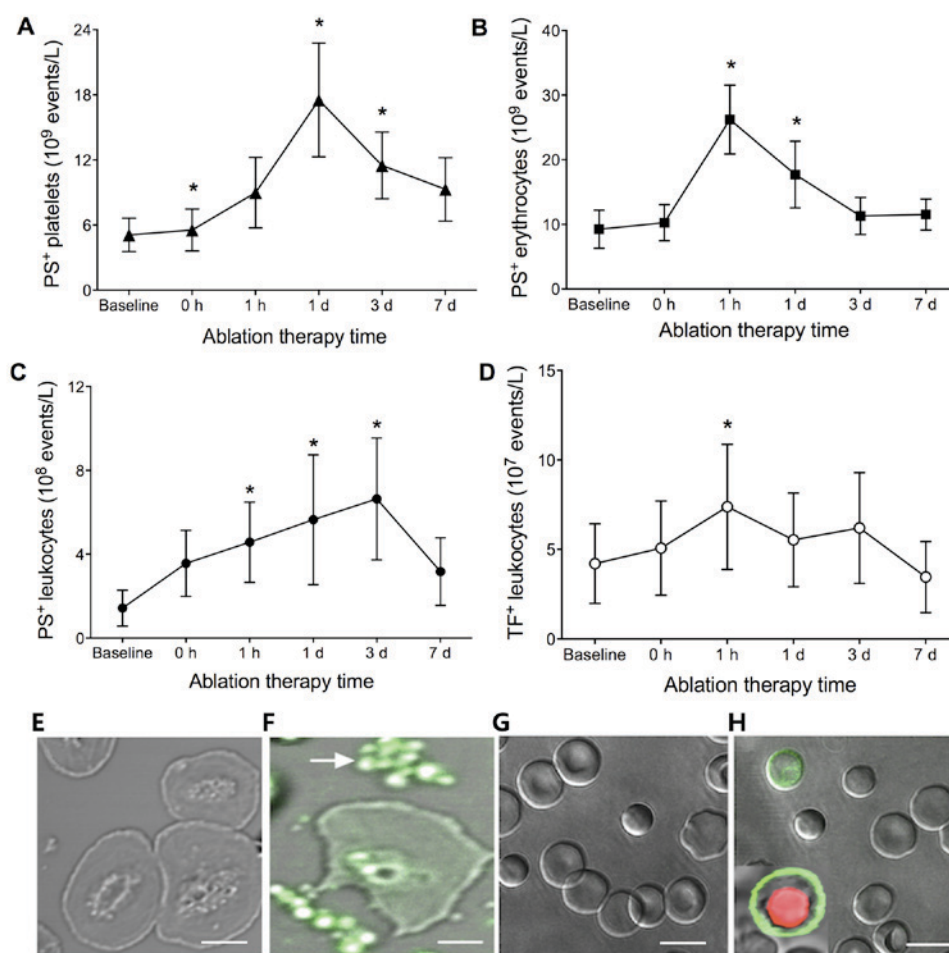


Figure 1. Numbers of PS⁺ (A) platelets, (B) erythrocytes and (C) leukocytes, and (D) TF⁺ leukocytes in patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 days and 7 days post-PVI. *P<0.01, compared with other time points. Fluorescence staining of PS⁺ (E) leukocytes at baseline and (F) 1 day post-PVI, and platelets and erythrocytes (G) at baseline and (H) 1 day post-PVI in patients with AF. No staining was observed at baseline. Green and red fluorescence represent positive staining of PS and propidium iodide, respectively. Scale bar=5 μm in E and F and 10 μm in G and H. AF, atrial fibrillation; PS, phosphatidylserine; TF, tissue factor; PVI, pulmonary vein isolation.

significant differences were found in the RMPs. No significant differences were found in the patients with paroxysmal and non-paroxysmal AF (data not shown). Following PVI, all

types of MPs, with the exception of TF-MPs (peak at 3 days) were significantly elevated with a peak at 1 day, followed by a decrease with intervention time (P<0.01; Table II).

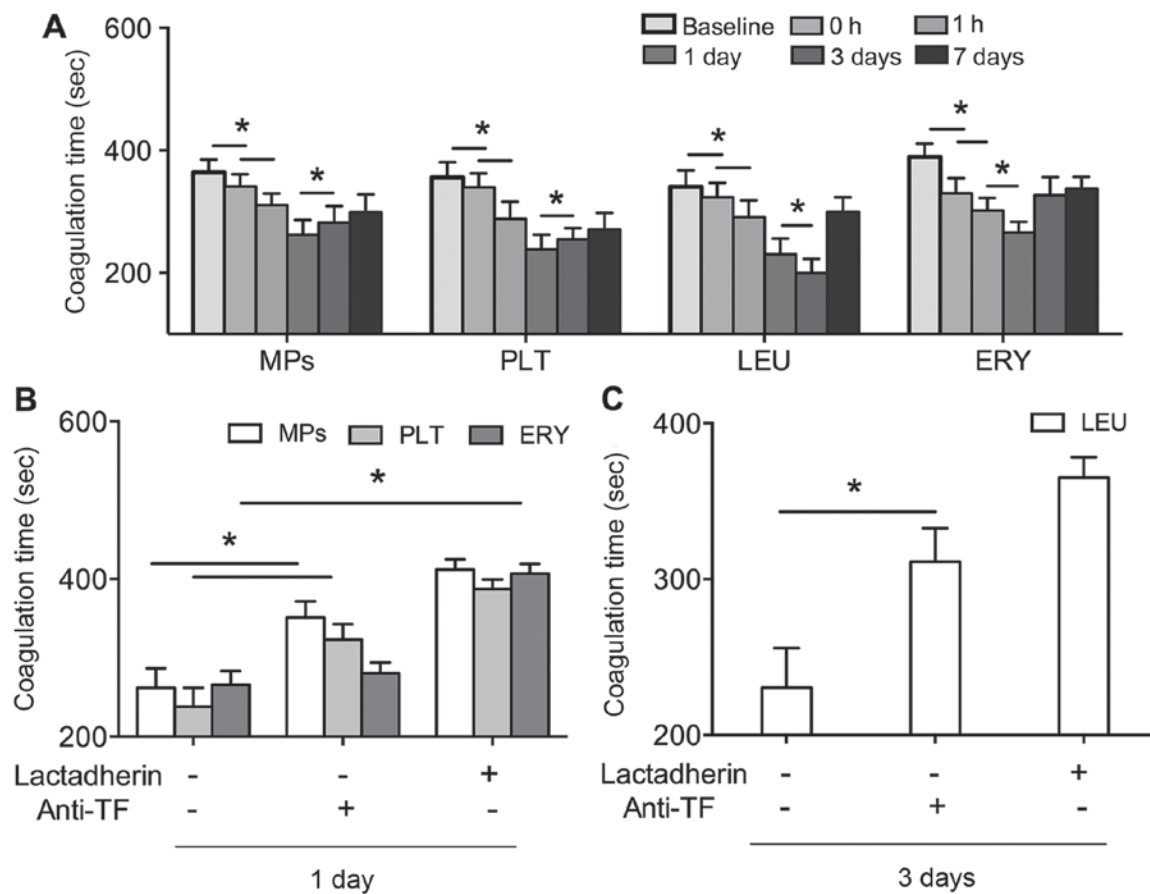


Figure 2. (A) Coagulation times of PLT, LEU, ERY and MPs in patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 day and 7 days post PVI. (B) Inhibitory effects of 128 nM lactadherin and 40 μ g/ml anti-TF on coagulation times of PLT, ERY and MPs 1 day post-PVI, and (C) LEU 3 days post-PVI in patients with AF. * P <0.01. AF, atrial fibrillation; PLT, platelet; LEU, leukocyte; ERY, erythrocyte; MPs, microparticles; TF, tissue factor; PVI, pulmonary vein isolation.

PCAs of PS⁺ blood cells and MPs. In order to reveal the PCA of PS in the patients with AF, the coagulation time of PS⁺ platelets, leukocytes, erythrocytes and MPs were evaluated. The coagulation times of these factors were significantly shortened by PVI, compared with those at baseline (P <0.01). The shortest coagulation time of the PS⁺ platelets, erythrocytes and MPs was observed at 1 day post-PVI, and at 3 days post-PVI for leukocytes (Fig. 2A). The effects of lactadherin and anti-TF on the coagulation times of these factors were also evaluated. As shown in Fig. 2B and C, the coagulation times of PS⁺ platelets, leukocytes, erythrocytes and MPs were all prolonged by lactadherin (P <0.01), which was close to baseline levels. Anti-TF also inhibited the coagulation times, although the inhibitory efficacy was lower, compared with that of lactadherin (P <0.01).

The formation of FXa and thrombin in PS⁺ platelets, leukocytes, erythrocytes and MPs were found to reveal alterations in PCA. The production of intrinsic and extrinsic FXa and thrombin in the PS⁺ platelets, leukocytes, erythrocytes and MPs were significantly increased and then reduced with intervention time post-PVI (P <0.01). At peak concentrations, the production of FXa and thrombin were 2-3-fold higher than at baseline (Fig. 3A-C). However, the contents of FXa and thrombin in platelets, leukocytes, erythrocytes and MPs were significantly inhibited by lactadherin (Fig. 3D-F). Anti-TF also inhibited the activity of extrinsic FXa in leukocytes and MPs (Fig. 3E).

Associations between the clinical characteristics of patients with AF and the numbers of PS⁺ blood cells/MPs. The associations between the numbers of PS⁺ blood cell/MPs and clinical characteristics of AF were analyzed using multiple linear regression analysis. As shown in Table III, the maximum power was significantly associated with PMPs (β =0.101; P =0.0009), and Hs-CRP was as a significant predictor of LMPs (β =0.325; P =0.038) and EMPs (β =0.114; P =0.004).

Prediction of ERAF by PS⁺ blood cells and MPs. The ROC was used to identify the role of PS⁺ blood cells and MPs in the prediction of ERAF at 1 day post-PVI treatment. As shown in Fig. 4A and B, the area under the curve (AUC) was >0.7 for PS⁺ leukocytes, LMPs and EMPs. The optimal cut-off values for the PS⁺ leukocytes, LMPs and EMPs were 568, 639 and 355/ μ l, respectively (Fig. 4A-C). In the prediction of ERAF, a sensitivity of 76.92% and specificity of 75% were exhibited for the PS⁺ leukocytes (AUC, 0.760; 95% CI, 0.587-0.932; P =0.001). Sensitivities of 69.23 and 92.31%, and specificities of 80 and 75% were exhibited for the LMPs (AUC, 0.792; 95% CI, 0.628-0.957; P <0.001) and EMPs (AUC, 0.835; 95% CI, 0.686-0.983; P <0.001), respectively.

The prognostic factors for ERAF were further analyzed using univariate and multivariate analyses. The results showed that hypertension (HR 1.96; 95% CI 0.75-5.14; P =0.047), non-paroxysmal AF (HR 2.35; 95% CI, 1.08-5.11; P =0.031),

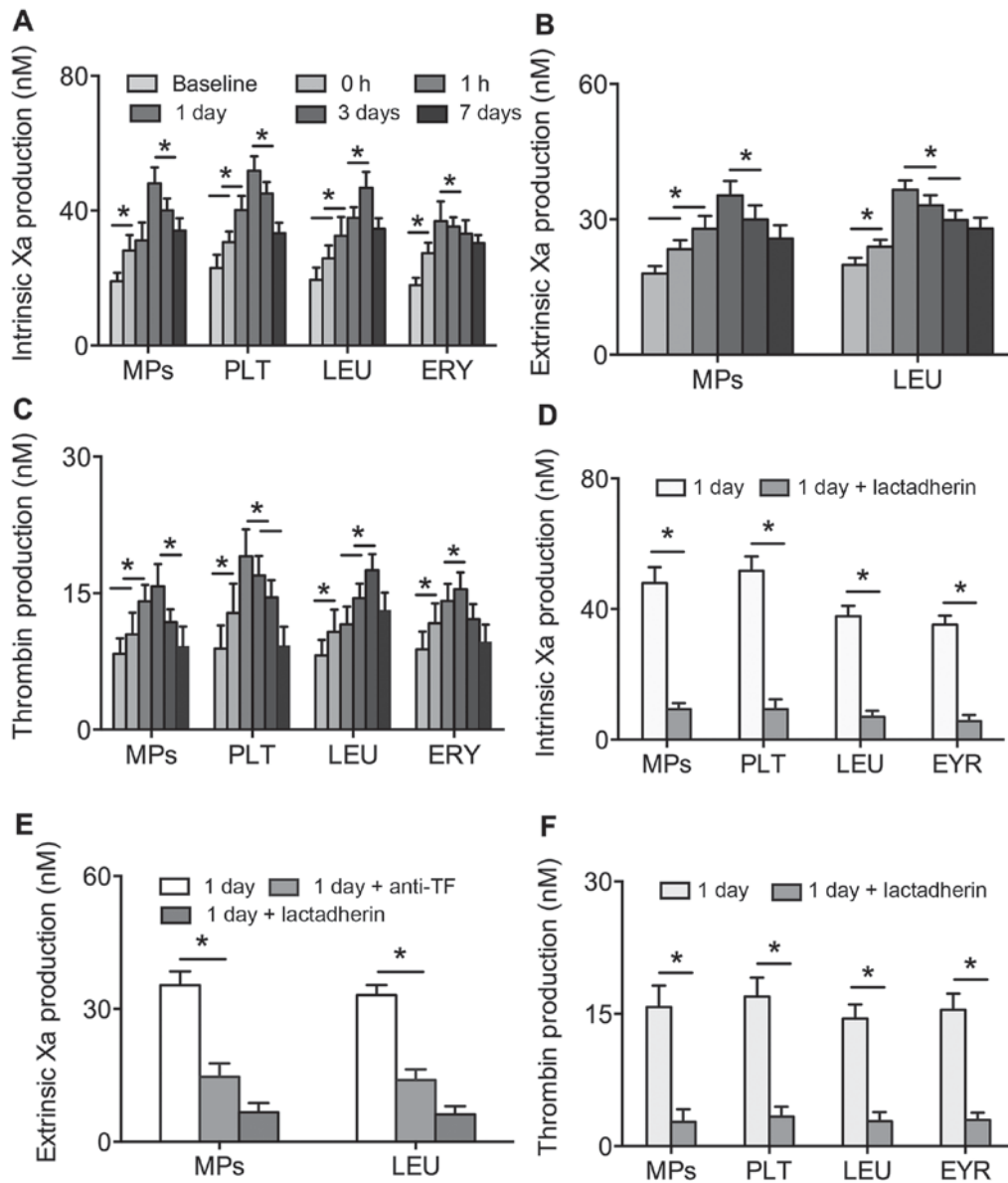


Figure 3. Production of (A) intrinsic FXa, (B) extrinsic FXa and (C) thrombin in PLT, LEU, ERY and MPs from patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 days and 7 days post-PVI. Inhibitory effects of 128 nM lactadherin and 40 µg/ml anti-TF on the production of (D) intrinsic FXa, (E) extrinsic FXa and (F) thrombin in PLT, LEU, ERY and MPs from patients with AF at 1 day post-PVI. *P<0.01. AF, atrial fibrillation; PLT, platelet; LEU, leukocyte; ERY, erythrocyte; MPs, microparticles; FXa, factor Xa; TF, tissue factor; PVI, pulmonary vein isolation.

hs-CRP (HR 5.47; 95% CI, 3.26-9.20; P=0.0001), PS⁺ leukocytes >568/µl (HR 2.17; 95% CI, 1.06-4.41; P=0.033), LMPs >639/µl (HR 1.29; 95% CI, 1.10-1.53; P=0.002) and EMPs >355/µl (HR 4.28; 95% CI, 2.03-9.03; P=0.0001) were risk factors for ERAF in the multivariate model. Furthermore, hs-CRP (HR 4.89; 95% CI, 2.37-6.28; P=0.0001), non-paroxysmal AF (HR 1.64; 95% CI, 1.03-2.61; P=0.03) and EMPs >355/µl (HR 4.92; 95% CI, 2.25-10.74; P=0.0001) were identified as independent predictors of ERAF (Table IV).

Discussion

PS is known to be important in the process of coagulation, and thrombosis is a risk factor in the treatment of AF by ablation (25,26). It has been reported that PS⁺ MPs exhibit marked procoagulant effects in patients with AF following

PVI (27). Similarly, the PCA of MPs in the patients with AF treated with PVI were significantly promoted by PS in the present study, which exhibited decreased coagulation time, and increased levels of FXa and thrombin. The present study is the first, to the best of our knowledge, to evaluate the procoagulant role of PS on blood cells. The results showed that PS exposure significantly reduced the coagulation time, and increased the production of FXa and thrombin in the blood cells of patients with AF treated with PVI. In addition, 80% of the PCA of the blood was inhibited by the intervention of lactadherin. These findings demonstrated that exposure of blood cells to PS increased the risk of thrombosis, and this effect may be caused by an additional procoagulant phospholipid surface for the assembly of thrombase complexes and thrombin generation in the circulation (28). In the clinical treatment of AF by PVI, the inhibition of PS⁺ blood cells

Table III. Multiple linear regression analysis of the associations between the number of PS⁺ blood cell/MPs and clinical characteristics of patients with AF.

Variable	Coefficient	PLT	LEU	ERY	PMPs	LMPs	RMPs	EMPs
Age	β -coefficient ^a	0.039	0.253	0.157	0.044	0.266	-0.027	0.001
	P-value	0.877	0.233	0.581	0.751	0.376	0.917	0.221
Male	β -coefficient	0.354	0.276	0.006	-0.018	-0.281	0.028	-0.047
	P-value	0.126	0.712	0.478	0.899	0.105	0.856	0.727
DM	β -coefficient	0.063	-0.091	-0.105	-0.028	0.000	-0.087	-0.071
	P-value	0.621	0.482	0.360	0.843	0.988	0.544	0.567
CAD	β -coefficient	0.020	0.142	0.010	0.047	-0.029	0.009	0.152
	P-value	0.881	0.292	0.894	0.290	0.831	0.143	0.239
Non-paroxysmal AF	β -coefficient	-0.063	-0.128	-0.028	0.021	-0.087	-0.152	0.189
	P-value	0.621	0.336	0.143	0.198	0.554	0.239	0.155
CHA ₂ DS ₂ -VASc \geq 2	β -coefficient	0.001	0.167	-0.042	-0.036	0.374	-0.276	-0.035
	P-value	0.246	0.868	0.162	0.216	0.321	0.427	0.793
Hs-CRP	β -coefficient	-0.126	0.115	0.103	-0.172	0.325	-0.190	0.114
	P-value	0.416	0.456	0.428	0.457	0.038 ^b	0.169	0.004 ^b
Total ablation time	β -coefficient	0.140	0.027	0.020	-0.207	-0.189	-0.060	0.611
	P-value	0.293	0.850	0.893	0.837	0.155	0.663	0.167
Maximum power	β -coefficient	0.103	0.158	-0.039	0.101	-0.014	0.096	0.003
	P-value	0.428	0.227	0.754	0.009 ^b	0.902	0.495	0.982
Baseline PS ⁺ PLT	β -coefficient	0.142	-	-	-	-	-	-
	P-value	0.292	-	-	-	-	-	-
Baseline PS ⁺ LEU	β -coefficient	-	0.128	-	-	-	-	-
	P-value	-	0.322	-	-	-	-	-
Baseline PS ⁺ ERY	β -coefficient	-	-	-0.006	-	-	-	-
	P-value	-	-	0.968	-	-	-	-
Baseline PS ⁺ PMPs	β -coefficient	-	-	-	0.178	-	-	-
	P-value	-	-	-	0.130	-	-	-
Baseline PS ⁺ LMPs	β -coefficient	-	-	-	-	-0.195	-	-
	P-value	-	-	-	-	0.183	-	-
Baseline PS ⁺ RMPs	β -coefficient	-	-	-	-	-	0.036	-
	P-value	-	-	-	-	-	0.812	-
Baseline PS ⁺ EMPs	β -coefficient	-	-	-	-	-	-	0.206
	P-value	-	-	-	-	-	-	0.108

^aNumbers are standardized β -coefficients. ^bP<0.05. AF, atrial fibrillation; PS, phosphatidylserine; PLT, platelets; LEU, leukocytes; ERY, erythrocytes; MPs, microparticles; PMPs, platelets-MPs; LMPs, leukocyte-MPs; EMPs, endothelial-MPs; RMPs, erythrocyte-MPs; DM, diabetes mellitus; CAD, coronary artery disease; Hs-CRP, high sensitivity C-reactive protein.

and MPs may be an effective method in the prevention of hypercoagulable states. TF on blood cells and MPs has also been suggested to be associated with increased PCA during PVI (29). In the present study, TF⁺ leukocytes were significantly increased at 1 h post-PVI, and anti-TF treatment significantly prolonged the coagulation time of the blood cells and MPs. This result indicated that thermally injured leukocytes induced by ablation increased the expression of TF (30). TF was also activated by PS residing on cell membranes and promoted the coagulation reaction (31).

Clinically, higher levels of MPs are found in patients with AF, and ablation is considered to affect the distribution of MPs (32). It has been reported that GPIb⁺ PMPs and CD11a⁺ LMPs are increased in patients with AF within

48 h following radiofrequency ablation or cryoablation (6). Consistent with previous findings, the present study showed that the numbers of all types of MPs were significantly increased by PVI in the patients with AF, which further indicated the activation of apoptosis in blood cells. However, associated studies on the associations between elevated PS⁺ MPs and clinical characteristics of patients with AF during PVI are limited. In the present study, the maximum power of PVI was an independent predictor of PS⁺ PMPs, which indicated the role of PMPs on the extent of tissue damage. In addition, the level of hs-CRP was correlated with PS⁺ LMPs and EMPs independently. This correlation suggested that the procoagulant state was associated with inflammation (33), potentially due to the activation of associated complements.

Table IV. Univariate and multivariate analyses of predictors of early AF recurrence in patients with AF treated with pulmonary veins isolation.

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	0.99 (0.96-1.02)	0.464	-	-
Male	1.25 (0.63-2.51)	0.526	-	-
History				
Hypertension	1.96 (0.75-5.14)	0.047 ^a	0.98 (0.96-1.01)	0.167
CAD	0.63 (0.26-1.90)	0.461	-	-
Non-paroxysmal AF	2.35 (1.08-5.11)	0.031 ^a	1.64 (1.03-2.61)	0.030 ^a
CHA ₂ DS ₂ -VASc ≥ 2	1.00 (1.00-1.01)	0.255	-	-
Transthoracic echocardiography				
LAD >40 mm	1.04 (0.98-1.11)	0.156	-	-
LVEF	1.62 (0.83-3.17)	0.161	-	-
Laboratory data				
Hs-CRP	5.47 (3.26-9.20)	<0.001 ^a	4.89 (2.37-6.28)	<0.001 ^a
PS ⁺ leukocytes >568/ μ l	2.17 (1.06-4.41)	0.033 ^a	0.84 (0.32-2.21)	0.721
PS ⁺ LMPs >639/ μ l	1.29 (1.10-1.53)	0.002 ^a	3.06 (0.62-14.98)	0.168
PS ⁺ EMPs >355/ μ l	4.28 (2.03-9.03)	<0.001 ^a	4.92 (2.25-10.74)	<0.001 ^a

^aP<0.05. AF, atrial fibrillation; CAD, coronary artery disease; LAD, left atrial diameter; LVEF, left ventricular ejection fraction; Hs-CRP, high sensitivity C-reactive protein; PS⁺, phosphatidylserine exposed; LMPs, leukocyte microparticles; EMPs, endothelial microparticles.

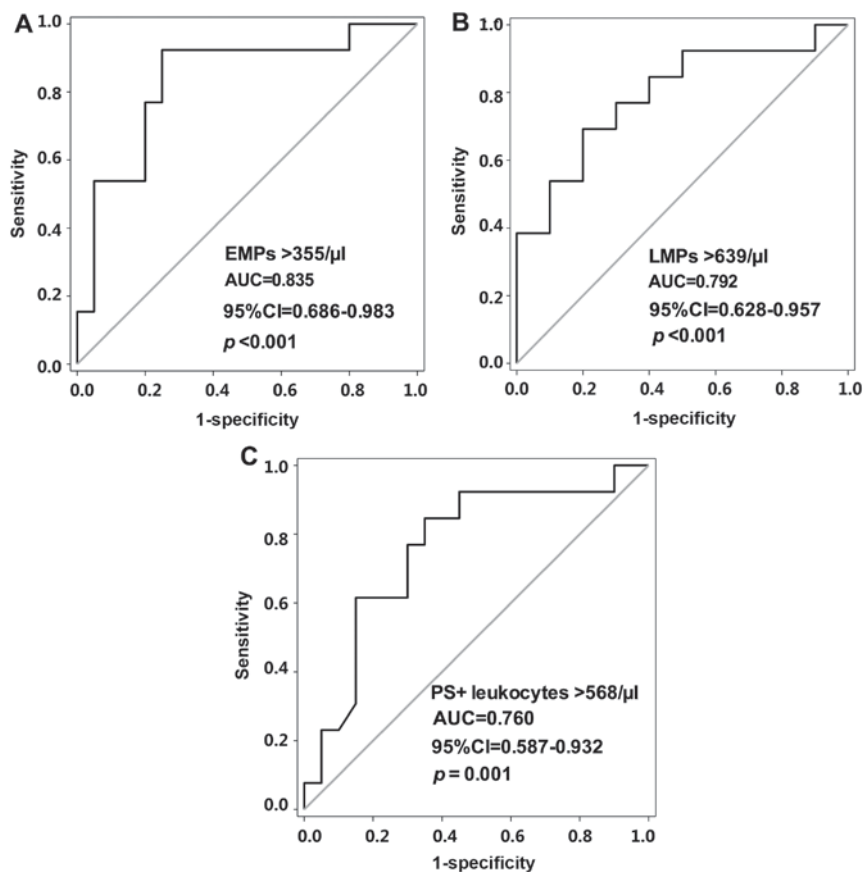


Figure 4. Prognostic role of PS⁺ (A) EMPs, (B) LMPs and (C) leukocytes on early recurrence AF in patients with AF at 1 day post-pulmonary vein isolation by receiver operating characteristic curve analysis. AF, atrial fibrillation; AUC, area under curve; CI, confidence interval; EMPs, endothelial-derived MPs; LMPs, leukocyte-derived MPs.

For example, the activation of cyclosporine 3 has been shown to induce MP shedding (34). However, non-paroxysmal AF and CHA₂DS₂-VASc scores ≥ 2 , which are particularly sensitive to disease severity in AF, were not found to be associated with MPs.

Although PVI is regarded as an effective and safe therapeutic option for patients with symptomatic and drug-refractory AF, ERAF occurs during follow-up (19). In the present study, PS⁺ EMPs $>355/\mu\text{l}$ was revealed to be a significant predictor of ERAF. This result supports the hypothesis that transient inflammation and procoagulant state following PVI-induced tissue damage contribute to the occurrences of ERAF (35). The preoperative hs-CRP level was also found to be an independent predictor of ERAF within 3 months following PVI. A high hs-CRP level has been associated with abnormal left atrial substrate and a high incidence of nonpulmonary vein AF sources, which contribute to the recurrence of AF (36), and the preoperative hs-CRP level has been associated with ERAF within 3 days following catheter ablation in patients with paroxysmal or persistent AF (37). The results of the present were consistent with previous studies, further demonstrating the predictive value of hs-CRP on ERAF during follow-up, and its association with inflammation and procoagulant activity. Therefore, the high prognostic values of hs-CRP and PS⁺ EMPs in ERAF were considered to be beneficial to the determination of AF in those not benefiting from PVI.

In conclusion, the present study demonstrated significant procoagulant effects exhibited by PS⁺ blood cells and MPs in patients with AF treated with PVI. Hs-CRP and EMPs $>355/\mu\text{l}$ were significant prognostic factors of ERAF during the follow-up period in patients with AF treated with PVI. However, limitations of the study included insufficient subjects, whereas bolus heparin and continuous aspirin treatment in patients may underestimate the activation of the coagulating cascade post-PVI. Therefore, further investigations on the procoagulant effect and long-term prognostic implications of PS⁺ blood cells and MPs in AF are required.

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