SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 8579-8588, 2017

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

HUAN MENG[1], JUNJIE KOU[1], RUISHUANG MA[2], WENBO DING[1], YAN KOU[3], MUHUA CAO[2], ZENGXIANG DONG[3], YAYAN BI[3], HEMANT S. THATTE[4] and JIALAN SHI[4]

[1]Department of Cardiology, The Second Hospital; Departments of [2]Hematology and [3]Cardiology, The First Hospital, Harbin Medical University, Harbin, Heilongjiang 150001, P.R. China; [4]Department of Surgery, VA Boston Healthcare System, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

Received February 15, 2016; Accepted January 27, 2017

DOI: 10.3892/mmr.2017.7763

**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/$\mu$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.

*Correspondence to:* Dr Junjie Kou, Department of Cardiology, The Second Hospital, Harbin Medical University, 246 Xuefu Road, Nangang, Harbin, Heilongjiang 150001, P.R. China
E-mail: junjiekou@163.com

Dr Yayan Bi, Department of Cardiology, The First Hospital, Harbin Medical University, 23 YouZheng Street, Nangang, Harbin, Heilongjiang 150001, P.R. China
E-mail: biyayan@163.com

*Key words:* pulmonary vein isolation, atrial fibrillation, phosphatidylserine, blood cells, microparticles

## 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,

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 $1x10^6$, were adjusted to a final volume of 45 $\mu$l 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 $\mu$l 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 (Al ex 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 $\mu$l suspensions of platelets, leukocytes and erythrocytes at a density of $1x10^6$ 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 $\mu$l 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 $\mu$g/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 $\mu$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 ± 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 $X^2$ 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 EARF 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), $CHA_2DS_2$-VASc score, left ventricular ejection fraction (LVEF) and left atrial diameter (LAD) were 2.5 (1.3-5.3) mg/l, 1.2±0.3, 61.18±10.22% and 34.97±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±39.01 min and the maximum power was 28.82±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±7.10 | 55.25±7.70 |
| Male/female | 25/15 | 34/22 |
| BMI ($kg/m^2$) | 23.43±3.23 | 23.75±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 ($10^3/\mu l$) | 6.74±1.35 | 6.91±1.62 |
| PT (sec) | 11.71±1.02 | 11.62±0.93 |
| APTT (sec) | 44.23±9.15 | 42.61±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 ± 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 thromboplasting 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 ($17.53±5.23x10^9$/l; P<0.01), as shown in Fig. 1A. $PS^+$ erythrocytes and leukocytes were increased with a peak at 1 h ($26.23±5.31x10^9$/l; P<0.001) and 3 days ($6.63±2.90x10^8$/l; P<0.01), respectively (Fig. 1B and C). $TF^+$ leukocytes were also increased with time, which peaked at 1 h ($7.23±3.20x10^8$/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.

| $PS^+$ (/$\mu$l) | 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±52b | 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. [a]P<0.01, compared with different time points; [b]P<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.

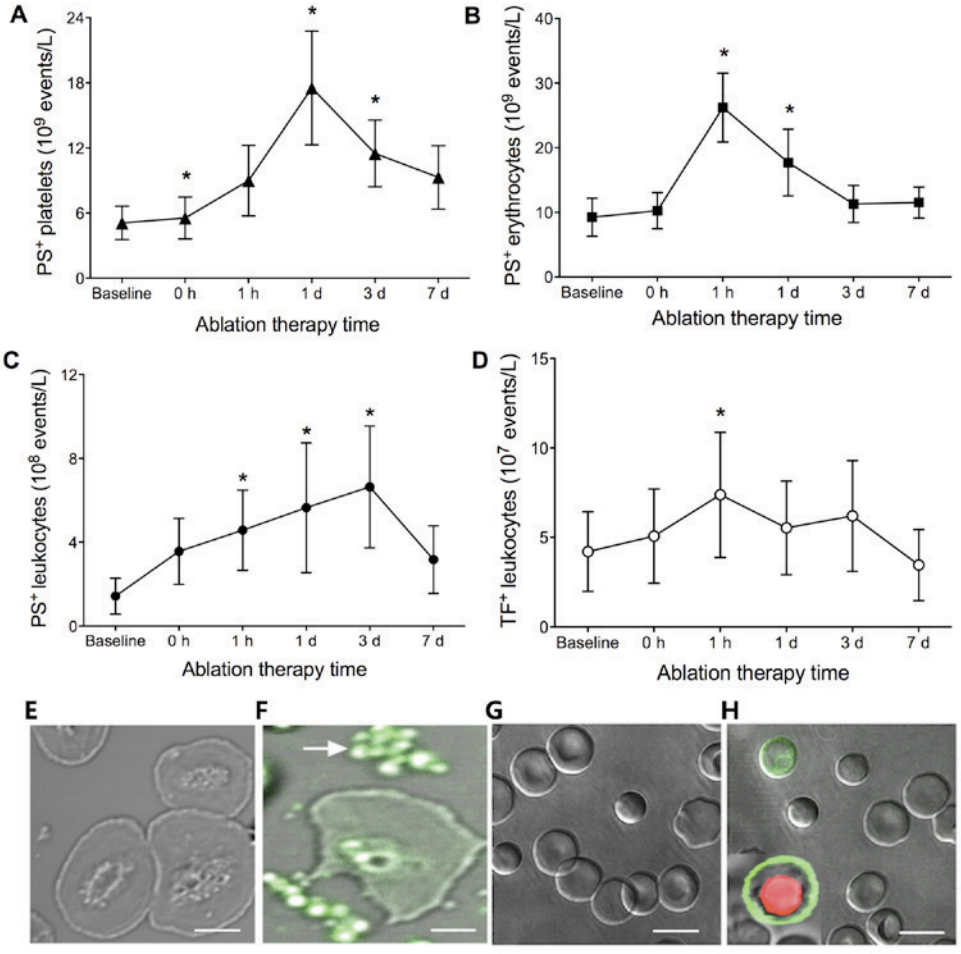


Figure 1. Numbers of $PS^+$ (A) platelets, (B) erythrocytes and (C) leukocytes, and (D) $TF^+$ leukocytes in patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 days and 7 days post-PVI. *P<0.01, compared with other time points. Fluorescence staining of $PS^+$ (E) leukocytes at baseline 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 $\mu$m in E and F and 10 $\mu$m 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).

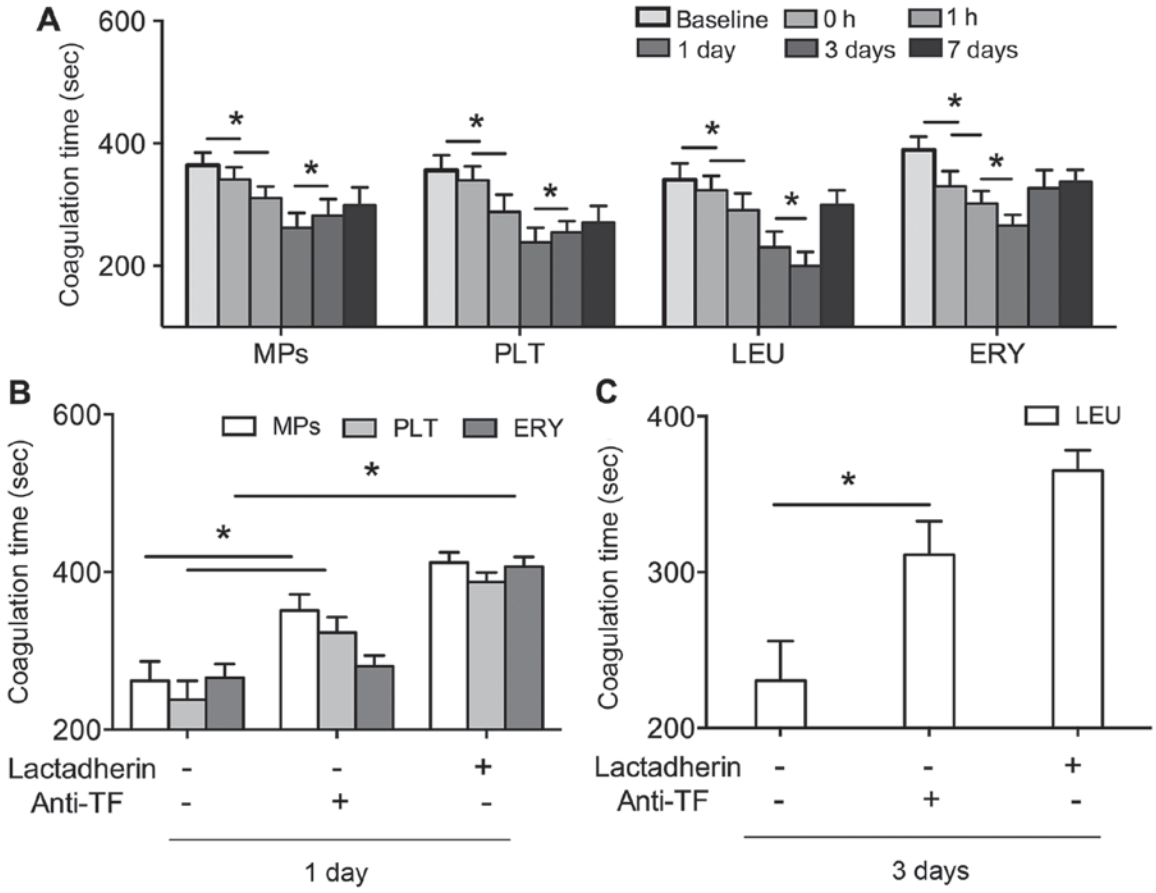


Figure 2. (A) Coagulation times of PLT, LEU, ERY and MPs in patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 day and 7 days post PVI. (B) Inhibitory effects of 128 nM lactadherin and 40 $\mu$g/ml anti-TF on coagulation times of PLT, ERY and MPs 1 day post-PVI, and (C) LEU 3 days post-PVI 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/$\mu$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),

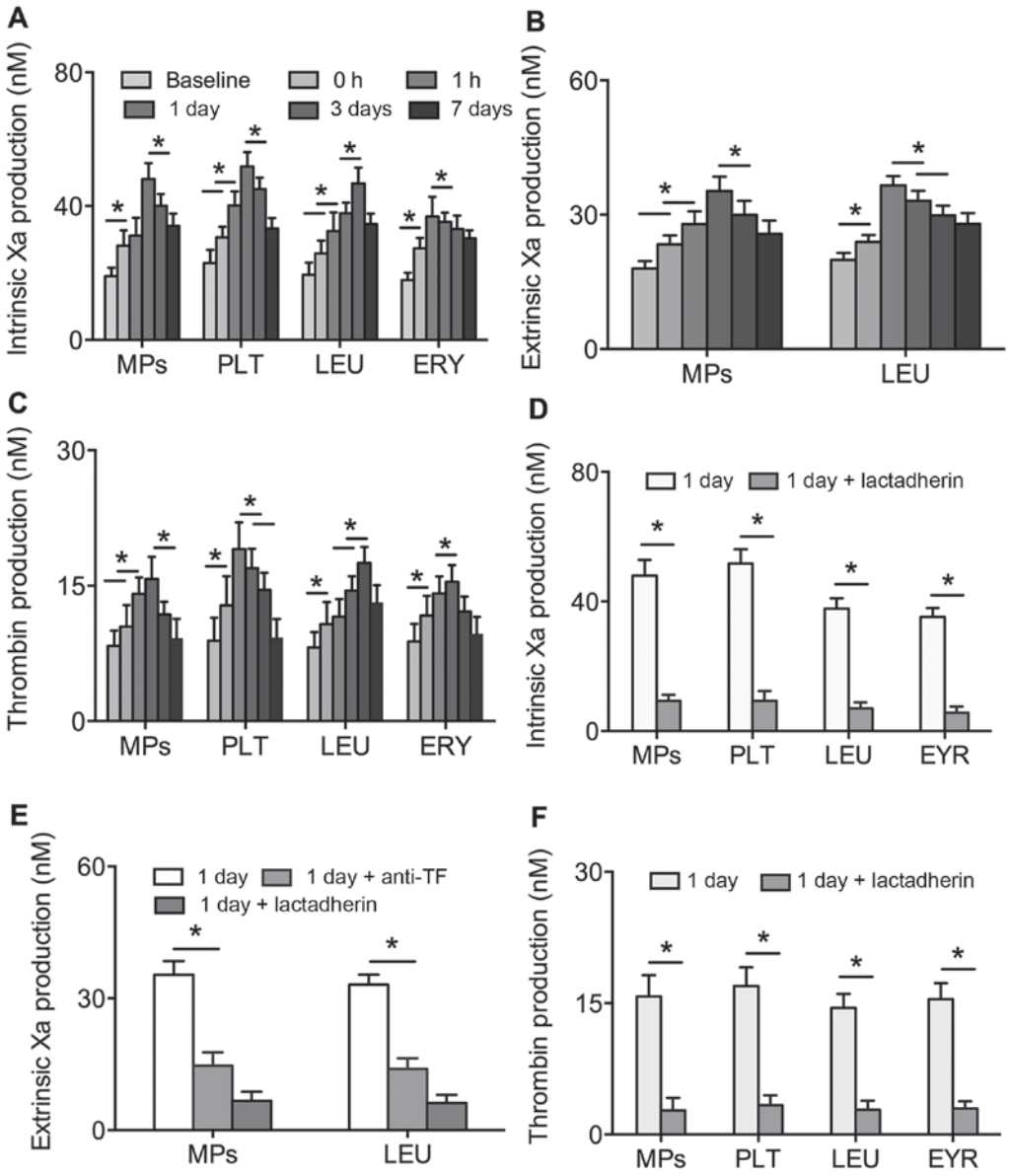


Figure 3. Production of (A) intrinsic FXa, (B) extrinsic FXa and (C) thrombin in PLT, LEU, ERY and MPs from patients with AF at baseline (prior to PVI), and 0, 1 h, 1 day, 3 days and 7 days post-PVI. Inhibitory effects of 128 nM lactadherin and 40 $\mu$g/ml anti-TF on the production of (D) intrinsic 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/$\mu$l (HR 2.17; 95% CI, 1.06-4.41; P=0.033), LMPs >639/$\mu$l (HR 1.29; 95% CI, 1.10-1.53; P=0.002) and EMPs >355/$\mu$l (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/$\mu$l (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 |
| $CHA_2DS_2$-VASc ≥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 |

[a]Numbers are standardized β-coefficients. [b]P<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 bean 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 be 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.

| | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| Variable | 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] |
| $CHA_2DS_2$-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/$\mu$l | 2.17 (1.06-4.41) | 0.033[a] | 0.84 (0.32-2.21) | 0.721 |
| $PS^+$ LMPs >639/$\mu$l | 1.29 (1.10-1.53) | 0.002[a] | 3.06 (0.62-14.98) | 0.168 |
| $PS^+$ EMPs >355/$\mu$l | 4.28 (2.03-9.03) | <0.001[a] | 4.92 (2.25-10.74) | <0.001[a] |

[a]P<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.

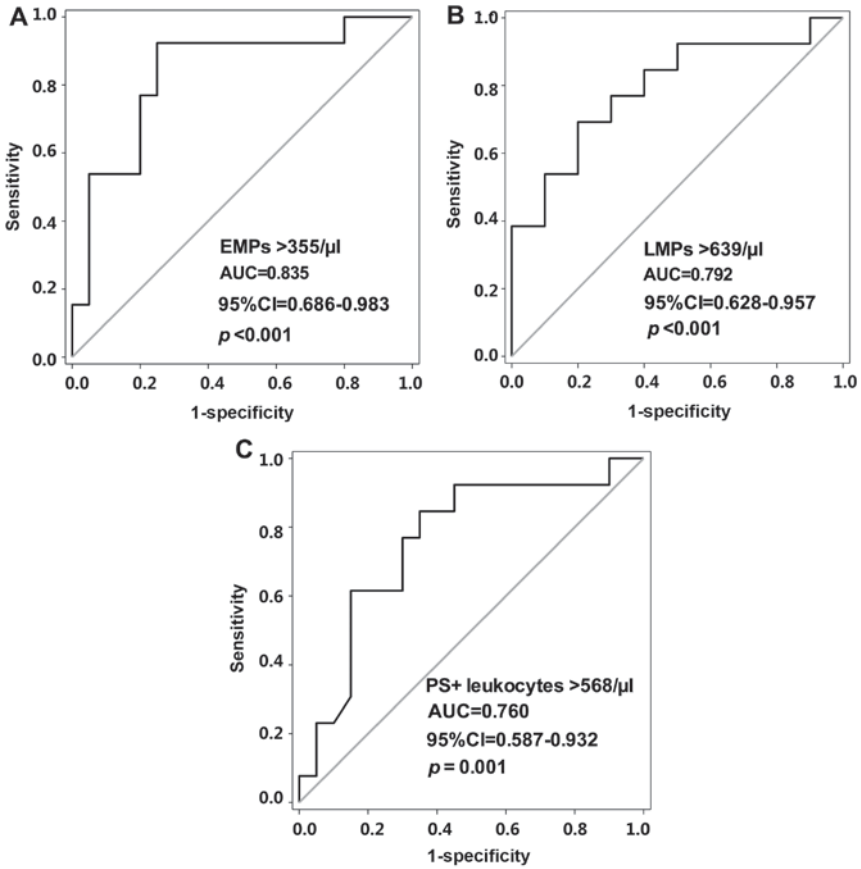


Figure 4. Prognostic role of $PS^+$ (A) EMPs, (B) LMPs and (C) leukocytes on early recurrence AF in patients with AF at 1 day post-pulmonary vein isolation by receiver operating characteristic curve analysis. AF, atrial fibrillation; AUC, area under curve; CI, confidence interval; EMPs, endothelial-derived 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 $CHA_2DS_2$-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/$\mu$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/$\mu$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.

## Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (grant nos. 81270588 and 81470301), the Natural Science Foundation of Heilongjiang Province (grant no. ZD2015020) and the Graduate Innovation Fund of Harbin Medical University (grant no. YJSCX2014-41HYD). The authors would like to thank Professor Bo Yu, Professor Jie Yuan, Professor Yong Sun, Ms. Xueqin Gao, Mr. Qinlong Tao and Ms. Na Han from the Department of Cardiology, The First Hospital, Harbin Medical University (Harbin, China) for sample collection.

## References

1. Lip GY and Lane DA: Stroke prevention in atrial fibrillation: A systematic review. JAMA 313: 1950-1962, 2015.
2. Calkins H, Kuck KH, Cappato R, Brugada J, Camm AJ, Chen SA, Crijns HJ, Damiano RJ Jr, Davies DW, DiMarco J, *et al*: 2012 HRS/EHRA/ECAS expert consensus statement on catheter and surgical ablation of atrial fibrillation: Recommendations for patient selection, procedural techniques, patient management and follow-up, definitions, endpoints, and research trial design. J Interv Card Electrophysiol 33: 171-257, 2012.
3. Noel P, Gregoire F, Capon A and Lehert P: Atrial fibrillation as a risk factor for deep venous thrombosis and pulmonary emboli in stroke patients. Stroke 22: 760-762, 1991.
4. Kiedrowicz RM, Kazmierczak J and Wielusinkski M: Left atrial massive thrombus formation on the transseptal sheath despite adequate anticoagulation with warfarin and heparin during pulmonary vein isolation. J Cardiovasc Electrophysiol 24: 1185, 2013.
5. Lim HS, Schultz C, Dang J, Alasady M, Lau DH, Brooks AG, Wong CX, Roberts-Thomson KC, Young GD, Worthley MI, *et al*: Time course of inflammation, myocardial injury, and prothrombotic response after radiofrequency catheter ablation for atrial fibrillation. Circ Arrhythm Electrophysiol 7: 83-89, 2014.
6. Herrera Siklódy C, Arentz T, Minners J, Jesel L, Stratz C, Valina CM, Weber R, Kalusche D, Toti F, Morel O and Trenk D: Cellular damage, platelet activation, and inflammatory response after pulmonary vein isolation: A randomized study comparing radiofrequency ablation with cryoablation. Heart Rhythm 9: 189-196, 2012.
7. Stazi A, Scalone G, Laurito M, Milo M, Pelargonio G, Narducci ML, Parrinello R, Figliozzi S, Bencardino G, Perna F, *et al*: Effect of remote ischemic preconditioning on platelet activation and reactivity induced by ablation for atrial fibrillation. Circulation 129: 11-17, 2014.
8. Haeusler KG, Kirchhof P and Endres M: Left atrial catheter ablation and ischemic stroke. Stroke 43: 265-270, 2012.
9. Gaita F, Caponi D, Pianelli M, Scaglione M, Toso E, Cesarani F, Boffano C, Gandini G, Valentini MC, De Ponti R, *et al*: Radiofrequency catheter ablation of atrial fibrillation: A cause of silent thromboembolism? Magnetic resonance imaging assessment of cerebral thromboembolism in patients undergoing ablation of atrial fibrillation. Circulation 122: 1667-1673, 2010.
10. Bulava A, Slavik L, Fiala M, Heinc P, Skvarilova M, Lukl J, Krcová V and Indrák K: Endothelial damage and activation of the hemostatic system during radiofrequency catheter isolation of pulmonary veins. J Interv Card Electrophysiol 10: 271-279, 2004.
11. Leventis PA and Grinstein S: The distribution and function of phosphatidylserine in cellular membranes. Annu Rev Biophys 39: 407-427, 2010.
12. Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A and Grinstein S: Membrane phosphatidylserine regulates surface charge and protein localization. Science 319: 210-213, 2008.
13. Rysavy NM, Shimoda LM, Dixon AM, Speck M, Stokes AJ, Turner H and Umemoto EY: Beyond apoptosis: The mechanism and function of phosphatidylserine asymmetry in the membrane of activating mast cells. Bioarchitecture 4: 127-137, 2014.
14. Tan X, Shi J, Fu Y, Gao C, Yang X, Li J, Wang W, Hou J, Li H and Zhou J: Role of erythrocytes and platelets in the hypercoagulable status in polycythemia vera through phosphatidylserine exposure and microparticle generation. Thromb Haemost 109: 1025-1032, 2013.
15. Tormoen GW, Recht O, Gruber A, Levine RL and McCarty OJ: Phosphatidylserine index as a marker of the procoagulant phenotype of acute myelogenous leukemia cells. Phys Biol 10: 056010, 2013.
16. Chen VM and Hogg PJ: Encryption and decryption of tissue factor. J Thromb Haemost 11 (Suppl 1): S277-S284, 2013.
17. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW and Radder JK: Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation 106: 2442-2447, 2002.
18. Geisbert TW, Young HA, Jahrling PB, Davis KJ, Kagan E and Hensley LE: Mechanisms underlying coagulation abnormalities in ebola hemorrhagic fever: Overexpression of tissue factor in primate monocytes/macrophages is a key event. J Infect Dis 188: 1618-1629, 2003.
19. Joshi S, Choi AD, Kamath GS, Raiszadeh F, Marrero D, Badheka A, Mittal S and Steinberg JS: Prevalence, predictors, and prognosis of atrial fibrillation early after pulmonary vein isolation: Findings from 3 months of continuous automatic ECG loop recordings. J Cardiovasc Electrophysiol 20: 1089-1094, 2009.
20. Takahashi Y, Takahashi A, Kuwahara T, Fujino T, Okubo K, Kusa S, Fujii A, Yagishita A, Miyazaki S, Nozato T, *et al*: Clinical characteristics of patients with persistent atrial fibrillation successfully treated by left atrial ablation. Circ Arrhythm Electrophysiol 3: 465-471, 2010.

21. Calkins H, Kuck KH, Cappato R, Brugada J, Camm AJ, Chen SA, Crijns HJ, Damiano RJ Jr, Davies DW, DiMarco J, *et al*: 2012 HRS/EHRA/ECAS expert consensus statement on catheter and surgical ablation of atrial fibrillation: Recommendations for patient selection, procedural techniques, patient management and follow-up, definitions, endpoints, and research trial design: A report of the heart rhythm society (HRS) task force on catheter and surgical ablation of atrial fibrillation. Developed in partnership with the European heart rhythm association (EHRA), a registered branch of the European society of cardiology (ESC) and the European cardiac arrhythmia society (ECAS); and in collaboration with the American college of cardiology (ACC), American heart association (AHA), the Asia pacific heart rhythm society (APHRS) and the society of thoracic surgeons (STS). Endorsed by the governing bodies of the American college of cardiology foundation, the American heart association, the European cardiac arrhythmia society, the European heart rhythm association, the society of thoracic surgeons, the Asia pacific heart rhythm society and the heart rhythm society. Heart Rhythm 9: 632-696.e21, 2012.
22. Gao C, Xie R, Yu C, Wang Q, Shi F, Yao C, Xie R, Zhou J, Gilbert GE and Shi J: Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticles release in patients with nephrotic syndrome. Thromb Haemost 107: 681-689, 2012.
23. Gao C, Xie R, Li W, Zhou J, Liu S, Cao F, Liu Y, Ma R, Si Y, Liu Y, *et al*: Endothelial cell phagocytosis of senescent neutrophils decreases procoagulant activity. Thromb Haemost 109: 1079-1090, 2013.
24. van Ierssel SH, Van Craenenbroeck EM, Conraads VM, Van Tendeloo VF, Vrints CJ, Jorens PG and Hoymans VY: Flow cytometric detection of endothelial microparticles (EMP): Effects of centrifugation and storage alter with the phenotype studied. Thromb Res 125: 332-339, 2010.
25. Spronk HM, ten Cate H and van der Meijden PE: Differential roles of tissue factor and phosphatidylserine in activation of coagulation. Thromb Res 133 (Suppl 1): S54-S56, 2014.
26. Kakkar AK, Mueller I, Bassand JP, Fitzmaurice DA, Goldhaber SZ, Goto S, Haas S, Hacke W, Lip GY, Mantovani LG, *et al*: Risk profiles and antithrombotic treatment of patients newly diagnosed with atrial fibrillation at risk of stroke: Perspectives from the international, observational, prospective GARFIELD registry. PLoS One 8: e63479, 2013.
27. Jesel L, Morel O, Pynn S, Radulescu B, Grunebaum L, Freyssinet JM, Ohlmann P, Bareiss P, Toti F and Chauvin M: Radiofrequency catheter ablation of atrial flutter induces the release of platelet and leukocyte-derived procoagulant microparticles and a prothrombotic state. Pacing Clin Electrophysiol 32: 193-200, 2009.
28. Puddu P, Puddu GM, Cravero E, Muscari S and Muscari A: The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. Can J Cardiol 26: 140-145, 2010.
29. Lwaleed BA, Breish MO, Birch BR, Chowdhary AP, Saad RA, Perigo O, Kazmi RS, Dusse LM and Cooper AJ: Tissue factor and tissue factor pathway inhibitor in women with a past history of preeclampsia: Implication for a hypercoagulable state post-pregnancy. Blood Coagul Fibrinolysis 25: 671-674, 2014.
30. Rao LV and Pendurthi UR: Regulation of tissue factor coagulant activity on cell surfaces. J Thromb Haemost 10: 2242-2253, 2012.
31. Bach RR: Tissue factor encryption. Arterioscler Thromb Vasc Biol 26: 456-461, 2006.
32. Jesel L, Abbas M, Toti F, Cohen A, Arentz T and Morel O: Microparticles in atrial fibrillation: A link between cell activation or apoptosis, tissue remodelling and thrombogenicity. Int J Cardiol 168: 660-669, 2013.
33. Kallergis EM, Manios EG, Kanoupakis EM, Mavrakis HE, Kolyvaki SG, Lyrarakis GM, Chlouverakis GI and Vardas PE: The role of the post-cardioversion time course of hs-CRP levels in clarifying the relationship between inflammation and persistence of atrial fibrillation. Heart 94: 200-204, 2008.
34. Renner B, Klawitter J, Goldberg R, McCullough JW, Ferreira VP, Cooper JE, Christians U and Thurman JM: Cyclosporine induces endothelial cell release of complement-activating microparticles. J Am Soc Nephrol 24: 1849-1862, 2013.
35. Grubman E, Pavri BB, Lyle S, Reynolds C, Denofrio D and Kocovic DZ: Histopathologic effects of radiofrequency catheter ablation in previously infarcted human myocardium. J Cardiovasc Electrophysiol 10: 336-342, 1999.
36. Lin YJ, Tsao HM, Chang SL, Lo LW, Tuan TC, Hu YF, Udyavar AR, Tsai WC, Chang CJ, Tai CT, *et al*: Prognostic implications of the high-sensitive C-reactive protein in the catheter ablation of atrial fibrillation. Am J Cardiol 105: 495-501, 2010.
37. Koyama T, Sekiguchi Y, Tada H, Arimoto T, Yamasaki H, Kuroki K, Machino T, Tajiri K, Zhu XD, Kanemoto M, *et al*: Comparison of characteristics and significance of immediate versus early versus no recurrence of atrial fibrillation after catheter ablation. Am J Cardiol 103: 1249-1254, 2009.