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Morphological determinators of platelet activation status in patients with atrial fibrillation

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ABSTRACT

Background: Stage of platelet activation is an important modulator of stroke risk associated with atrial fibrillation (AF). However, factors determining such activation status of thrombocytes in patients with AF are still not well studied.

Methods and results: We enrolled 83 patients (mean age 61 ± 10 years, 61% male, mean CHA_2DS_2-VASc 2.1 ± 1.4) with paroxysmal (75%) or persistent (25%) AF admitted for catheter ablation. Blood samples were collected directly from the left atrium (LA) and platelet activation status was measured by means of flow cytometric assessment in whole blood and light transmission aggregometry (LTA) in unstimulated and Thrombin-receptor-activated-peptide-6 (TRAP-6)-stimulated platelet rich plasma. In flow cytometry, we measured fractions of platelet microparticles and aggregates as well as P-selectin expression on platelets' surfaces. LTA findings are expressed as maximal aggregation (MA), primary slope (PS) and area under curve (AUC). Cardiac anatomy has been assessed by means of echocardiography and magnetic resonance imaging.

Left atrial appendage (LAA) volume, but not LAA morphology nor morphological and functional parameters describing LA, was significantly correlated with increased pre-activation of platelets ($R = 0.224$, $p = 0.043$) and consecutive reduced response to TRAP-6 ($R = 0.231$, $p = 0.037$) measured by P-selectin expression in flow cytometry. Similarly, a reduced response to TRAP-6 in patients with larger LAA volume (PS: $R = -0.240$; $p = 0.042$; AUC: $R = -0.244$; $p = 0.035$; MA: $R = -0.270$; $p = 0.019$) as well as with heart failure (PS 54.75 vs 71.45, $p = 0.026$) was observed in LTA.

Conclusion: In patients with AF, LAA volume correlates with extent of platelet activation status, this effect is aggravated in patients with heart failure.

1. Introduction

Thromboembolic events are complications of major clinical impact in patients with atrial fibrillation (AF). On average, the attributable stroke risk associated with AF is described as being about 5% per year, but may vary from 1.5–23.5% depending on age and cardiovascular comorbidities [1]. On a molecular level, multiple markers of coagulation, fibrinolysis (fibrinogen, D-dimer, factor VIIIc, tissue plasminogen activator, thrombin-antithrombin complex, prothrombin fragments 1 + 2), and endothelial lesions (von Willebrand factor, thrombomodulin) were shown to be elevated in patients with AF compared to sinus rhythm (SR) [2–4]. Moreover, measures of platelet activation (platelet factor 4 [PF4], beta-thromboglobulin, P-selectin) were also shown to be elevated in patients with stroke and non-valvular AF [3,5,6]. However, morphological factors that modulate individual baseline platelet activation level, and thus stroke risk, have not been well studied in a cohort of AF patients. To better understand the mechanisms that underlie the increased stroke risk attributable to platelet activation status, we sought to assess the individual factors determining platelet activation status in AF.

2. Methods

2.1. Inclusion criteria

We enrolled 83 consecutives patients from March 2016 to August 2017 with symptomatic paroxysmal or persistent AF admitted to Heart Center Leipzig for catheter ablation. Patients with previous AF ablation procedures including surgical ablation, medication with antiplatelet agents (ASS, P2Y12-inhibitors), or any contraindications for cardiovascular magnetic resonance (CMR) imaging or the application of a gadolinium-

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Fig. 1. Model graph for LTA: Light transmission in TRAP-6-activated PRP is measured for 10 min and recorded as a curve; results are expressed as primary slope (PS), area under curve (AUC), and % maximal aggregation (MA).

containing contrast agent were excluded. Paroxysmal and persistent AF were defined according to current guidelines [7]. The study was approved by the Institutional Ethics Committee and conforms with the Declaration of Helsinki. All patients provided written agreement concerning data collection and analysis.

2.2. Blood sampling

Blood samples from the left atrium (LA) were drawn from a 11F transseptal catheter sheath after completing transseptal puncture. Transseptal access and catheter navigation were performed with a steerable sheath (Agilis, Abbott, St. Paul, MN, USA). After discarding 20 ml, another 20 ml were aspirated avoiding shear stress and filled into two 3.0 ml sodium citrate tubes (Sarstedt AG & Co., Nümbrecht, Germany), which were used for platelet function tests. Blood samples were handled carefully, kept at room temperature, transported in a non-mechanical way to a GCP- and CE certified laboratory, and processed within a maximum of 90 min [8].

2.3. Flow cytometry

For the measurement of the P-selectin expression-density, platelets were marked with platelet-specific monoclonal antibody CD41-PE and activation-specific antibody CD62P-FITC ex vivo and after Thrombin receptor-activated peptide-6 (TRAP-6)-stimulation. Fluorescing intensity of CD62P-FITC-positive platelets was measured by flow cytometer. All FACS experiments were performed in whole blood according to interdisciplinary S2K-AMWF-Guidelines [9]. Healthy control donor blood was analyzed for validation of staining and FACS once a week. Depending on platelet count, blood samples were diluted with HBSS-buffer (37 °C) to reach a concentration of 20,000 platelets/μl. 36 μl diluted blood were incubated with 4 μl HBSS-buffer in first tube or 4 μl 100 μM of strong platelet activator TRAP-6 (Loxo, Dossenheim, Germany) in second tube for 10 min at 37 °C. Then 20 μl mix of PE-labeled antibody against Gp IIb/IIIa (CD41-PE; Beckmann Coulter, Krefeld, Germany) and FITC-labeled P-selectin (CD62P; Beckmann Coulter, Krefeld, Germany) were added and incubated for 5 min at 37 °C again. By adding 2 ml cold (4 °C) HBSS-buffer, activation was stopped. Cell fixation was not conducted to avoid falsification of results and not required as all measurements were performed in the predefined time limit. Mean fluorescing intensity of CD62P positive platelets was measured using Beckmann Coulter CYTOMICS FC500 flow cytometer. Data was expressed in logarithmic scale and presented as fluorescing units (FU). Platelet microparticles and aggregates were counted automatically and data was expressed in % of all CD41-expressing cells. For better inter-individual comparability, platelet activation status was further calculated as the ratio of unstimulated and TRAP-6-stimulated platelets and noted in percent of TRAP-6 response which was set as maximum activation.

2.4. Light transmission aggregometry (LTA)

To extract platelet rich plasma (PRP), citrated whole blood specimens were centrifuged at 150*g* for 10 min at room temperature. If erythrocytes remained, supernatant was removed by using a pipette and centrifuged again at 150*g* for 5 min, and no erythrocyte lysis was required. PRP was further portioned and centrifuged at 3100*g* for 20 min to gain platelet poor plasma (PPP). Measurements were performed in PRP at a predefined and validated range of platelet count (150–450 $\times$ 10^6/ml). If platelet count was above 450 $\times$ 10^6/ml, PRP was diluted with autologous PPP until reaching the normal range. 2 $\times$ 180 μl PRP was pipetted in cuvettes and incubated for 3 min at 37 °C. PPP was calibrated as 100% light transmission and set as maximum aggregation [10]. PRP cuvettes were inserted into PAP-8E (möLab, Langenfeld, Germany) aggregometer and automatic recording started after administration of 20 μl of TRAP-6 into the measuring channel. Measured curves were registered for 10 min. Results were presented in primary slope (PS) of light transmission curve, % maximal aggregation (MA), and area under the curve (AUC). LTA model graph can be found in Fig. 1.

2.5. Echocardiography

All patients underwent a comprehensive Doppler and M-mode transthoracic echocardiographic according to the recommendations of the American Society of Echocardiography [11]. The Vivid-7 System and Vivid-9 System (Vingmed/General Electric, Milwaukee, WI, USA) with a 3.6-MHz probe were used for echocardiography. The largest LA dimension was measured in the two-dimensional (2D) parasternal long axis view at end systole and left ventricular ejection fraction (LVEF) was assessed by monoplane Simpson's method from the apical 4-chamber view [12].

2.6. CMR

All CMR examinations were performed on a 1.5-T MRI system (Ingenia, Philips Healthcare, Best, Netherlands) equipped with a standard 28 channel array coil with full in-coil signal digitalization combined with optical transmission. For cine imaging, a balanced steady-state free procession (bSSFP) sequence with retrospective gating (>30 phases per cardiac cycle) was used during end-expiratory breath holding. Cine images were acquired in standard cardiac geometries (2-, 3- and 4-chamber views and a short axis stack covering the entire left ventricle from the mitral valve to the apex). A 3-dimensional contrast-enhanced angiography of the left atrium and the pulmonary veins was performed using a contrast-enhanced, non-ECG triggered spoiled gradient echo sequence (intravenous bolus of 0.1 mmol/kg gadolinium-DTPA, injection rate of 4.0 ml/s).

2.7. Morphological description of left atrium and left atrium appendage

CMR data were reviewed using multiplanar reconstruction, maximum intensity projection, and 3D volume rendering in a specialized image processing software (EnSite Verismo, Abbott, MN, USA). LA and left atrial appendage (LAA) volume were measured. Three physicians experienced with cardiovascular imaging and blinded to the clinical data of the patients analyzed LAA morphology. In case of disagreement, a further expert physician was consulted and based on common consent a decision was made. LAA morphology was classified in 4 types, as previously defined: Cactus, Chicken Wing, Cauliflower, and Windsock [13]. Pattern of pulmonary vein (PV) drainage was assessed in order to detect common ostium as well as accessory veins [14]. In all patients, LA myocardium was checked for low voltage areas using high density direct contact electroanatomical mapping during catheter ablation. Low voltage areas were defined as spots containing ≥3 proximate points with measured voltage <0.5 mV [15].

2.8. Statistical analysis

Continuous variables are given as mean ± SD and categorical variables as frequencies. The correlation between platelet activation status and continuous variables was assessed with Pearson or Spearman correlation statistic depending on statistical distribution and with Eta statistic or *t*-test for nominal variables. A p-value of <0.05 was considered statistically significant. Analysis was performed with SPSS (version 20.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Patient characteristics

The population consists of 83 patients (61.4% male) with history of paroxysmal (74.7%) or persistent AF (25.3%). Mean age was 61.2 ± 10.6 years with a median duration of AF history of 35.3 months. At the time of blood withdrawal, 60 patients (72.3%) had SR, 20 (24.1%) patients AF, two (2.4%) patients atrial flutter, and another one (1.2%) a ventricular paced rhythm. Diabetes was found in 14 (16.9%) patients, 4 (4.8%) patients had a history of previous stroke or transient ischemic attack, and mean BMI was 28.8 ± 4.9 kg/m^2. CHA2DS2-VASc-Score varied between 0 and 5 with mean of 2.1 ± 1.4. Most patients were treated with direct oral anticoagulants (69.9%) while 20.5% were on vitamin K antagonists. Antiarrhythmic medication was found in 33 patients (39.7%), most frequently with flecainide (14.5%). 64 patients were treated with beta-blockers (77.1%).

Detailed patient characteristics can be found in Table 1.

Table 1
Patient characteristics.

Characteristic	
Age – yr – Mean (SD)	61.2 (10.6)
Male sex – no. (%)	51 (61.4)
Body-mass index – Mean (SD)	28.8 (4.9)
Type of AF – no. (%)	
Paroxysmal AF	62 (74.7)
Persistent AF	21 (25.3)
History of AF – months – Mean (SD)	35.3 (39.6)
Medical history – no. (%)	
Hypertension	65 (78.3)
Coronary artery disease	4 (4.8)
Stroke or TIA	4 (4.8)
Diabetes	14 (16.9)
CHA_2DS_2-VASc-Score – no. (%)	
0	12 (14.5)
1	16 (19.3)
2	20 (24.1)
3	21 (25.3)
4	12 (14.5)
5	2 (2.4)
Drugs – no. (%)	
β-Blocker	64 (77.1)
ACE inhibitors	46 (55.4)
Statin	18 (21.7)
Calcium channel blockers	16 (19.3)
Flecainide	12 (14.5)
Amiodarone	3 (3.6)
Propafenone	1 (1.2)
Dronedarone	8 (9.6)
Digitalis	9 (10.8)
Anticoagulation – no. (%)	
Low molecular weight heparin	1 (1.2)
Phenprocoumon	17 (20.5)
Dabigatran	6 (7.2)
Rivaroxaban	22 (26.5)
Apixaban	23 (27.7)
Edoxabane	7 (8.4)
GFR – ml/min/1,73 m^2 – Mean (SD)	78.4 (18.0)
Left ventricular ejection fraction – % – Mean (SD)	56.3 (8.3)
Aorta diameter – mm – Mean (SD)	34.2 (4.7)
Interventricular septum – mm – Mean (SD)	12.0 (1.6)
LA dimension – mm – Mean (SD)	43.2 (7.0)
LA area – mm^2 – Mean (SD)	26.1 (6.5)
LA volume – ml – Mean (SD)	103.7 (34.4)
LAA volume – ml – Mean (SD)	6.3 (3.1)
LAA morphology – no. (%)	
Chicken wing	24 (28.9)
Cactus	29 (34.9)
Windsock	16(19.3)
Cauliflower	13 (15.7)
LA characteristics – no. (%)	
Common ostium	7 (8.4)
Accessory pulmonary vein	21 (25.3)
Heart rhythm during blood withdrawal – no. (%)	
Sinus rhythm	60 (72.3)
Atrial fibrillation	20 (24.1)
Atrial flutter	2 (2.4)
Paced rhythm	1 (1.2)
Heart rate during blood withdrawal – bpm – Mean (SD)	73.0 (14.5)
Low voltage – no. (%)	10 (12.0)

3.2. Characteristics of LA and LAA parameters

Mean LA volume measured in CMR was 103.7 ± 34.4 ml, while mean LA diameter obtained from echocardiographic study was 43.2 ± 7.0 mm. Mean LAA volume was 6.3 ± 3.1 ml. In 24 (28.9%) patients, LAA was classified as a chicken wing morphology, in 29 (34.9%) patients, cactus morphology could be identified, 16 (19.3%) patients appeared as windsock morphology, and 13 (15.7%) patients had a cauliflower-LAA-morphology. Common ostium was detected in 7 patients (8.4%) while 21 (25.3%) had an accessory pulmonary vein. Low voltage areas were localized in 10 patients (12%).

Table 2
Flow cytometry correlation data.

Correlation parameter	R	P
P-selectin expression in unstimulated platelets (1626.19 ± 1246.03)		
LAA volume	0.224	0.043
PMP unstimulated	0.368	0.001
P-selectin expression ratio unstimulated/TRAP-6-stimulated (12.25% ± 8.86%)		
LAA volume	0.231	0.037
Platelet aggregates in unstimulated blood (5.17% ± 1.96%)		
LA dimension (TTE)	0.395	0.021
LA volume	0.248	0.026
Age	−0.307	0.005

3.3. Platelet activation status

In flow cytometry, baseline platelet activation was 1626.19 ± 1246.03 FU in unstimulated platelets and 14,004.89 ± 3884.40 FU in TRAP-6-stimulated platelets, giving an unstimulated/TRAP-6 stimulated ratio of 12.25% ± 8.86%. LAA volume correlated significantly with an increase of P-selectin expression ($R = 0.224$, $p = 0.043$) and a decrease of response to TRAP-6 stimulation ($R = 0.231$, $p = 0.037$). Furthermore, patients with mitral insufficiency showed lower P-selectin expression after TRAP-6 stimulation (12,939.63 ± 3564.61 FU vs 15,150.05 ± 3929.97 FU).

Fraction of PMP in unstimulated blood was 1.77% ± 1.16% and 2.79% ± 1.67% after TRAP-6 stimulation, while diabetics had a significantly higher PMP fraction (1.86% ± 1.23% vs 1.31% ± 0.60%; $p = 0.016$).

In LTA after TRAP-6-stimulation, a significant negative correlation of LAA volume with the following parameters was found: PS (70.5 ± 14.7; $R = -0.240$; $p = 0.042$), AUC (815.3 ± 154.7; $R = -0.244$; $p = 0.035$) and MA (92.0% ± 16.0%; $r = -0.270$; $p = 0.019$). LTA parameters were further influenced by glomerular filtration rate (PS: $R = 0.242$; $p = 0.039$; AUC: $R = 0.244$; $p = 0.034$; MA: $R = 0.264$; $p = 0.021$) and ejection fraction (PS: $R = 0.246$; $p = 0.036$; AUC: $R = 0.243$; $p = 0.034$). Moreover, a reduced response to TRAP-6 was detected in patients with congestive heart failure (PS: 54.75 ± 2.06 vs 71.45 ± 14.55, $p = 0.026$) and mitral insufficiency (PS: 66.4 ± 13.4 vs 75.4 ± 14.8; $p = 0.009$; MA: 88.3 ± 14.1% vs 96.2 ± 17.1%; $p = 0.030$). Further data for flow cytometry and LTA can be found in Tables 2 and 3.

4. Discussion

4.1. Main findings

To the best of our knowledge, this is the first study analyzing comprehensive factors modulating platelet activation status in patients with AF using blood samples drained directly from the LA. In this analysis, the LAA volume was the only independent morphological

Table 3
LTA correlation data.

Correlation parameter	R	P
Primary slope (70.5 ± 14.7)		
LAA volume	−0.240	0.042
EF	0.246	0.036
GFR	0.242	0.039
AUC (815.3 ± 154.7)		
LAA volume	−0.244	0.035
EF	0.243	0.034
GFR	0.244	0.034
MA (92.0% ± 16.0%)		
LAA volume	−0.270	0.019
GFR	0.264	0.021

modulator of platelet activation measured by LTA and flow cytometric assessment of P-selectin expression in patients with AF.

4.2. Platelet activation status and AF

Platelet activation status in AF patients is a controversial issue and its significance in AF-related thromboembolism is not fully understood. Nevertheless, its participation in prothrombotic processes cannot be denied [16]. Increased levels of molecular markers of coagulation, fibrinolysis, endothelial damage, and platelet activation in AF compared to SR were identified in multiple studies [2–4,6,17–19]. Furthermore, inflammation and oxidative stress typical for AF can also aggravate prothrombotic state of platelets [20–23] which can be assessed by several parameters. Platelet activation markers beta-thromboglobulin and PF4 are described to be elevated in patients with paroxysmal AF along with significant increase 12 h after acute onset of AF [3,6].

Platelet P-selectin, another indicator of platelet activation status, has been shown to be severely elevated in patients with AF [5,19,24]. Interestingly, this mediator of platelet-neutrophil-interaction is unaffected by antithrombotic therapy and has been identified as a possible risk factor for silent stroke [25]. Moreover, an increased expression of P-selectin on platelets' surface in AF patients is linked to inhibition of NO-synthesis by endothelial damage and the arrhythmia itself [5,25,26]. We showed a higher level of P-selectin expression in AF patients with larger LAA volumes. Furthermore, in patients with larger LAA, we observed a decreased platelet reactivity to TRAP-6 which can be explained by pre-activation of platelets and consecutive reduced response to TRAP-6.

Nevertheless, the relevance of P-selectin for AF-related stroke risk is still not entirely proven and challenged by some authors [2,27].

Recently, PMP, membrane vesicles originating from activated platelets mediating hemostasis and thrombosis [28], became a focus of investigations since its elevated level has been found in stroke patients [29,30]. Also, in patients with AF, a higher level of PMP has been detected [31,32]. Moreover, an emerging role of PMP in thromboembolism in AF patients was presented in different studies [28,33].

Although a correlation between PMP and morphological factors was not evident based on data in our study, we did observe a larger percentage of PMP in patients with diabetes which is consistent to the analysis of Li et al. [34].

Nevertheless, the significance of flow cytometry data on PMP limited to their fractions in CD41b-positive cells is questionable since it lacks blood levels and functional testing, such as expression of phosphatidylserine, which has recently been addressed in this journal to play an important role in procoagulant activity in AF [33].

To estimate platelet activation status, we further used LTA in PRP to simulate in vitro processes mediating thromboembolism in AF patients. Platelet stimulation by TRAP-6 results in liberation of platelets' intracellular alpha granules via selectively activating platelet activation receptor 1 [35,36]. We found a negative correlation of LAA volume with PS, AUC, and MA after TRAP-6-stimulation in LTA. We assume that larger LAA volumes induce a higher degree of pre-activation resulting in an amplified desensitization and more decreased response to TRAP-6. Similar, reduced platelet aggregation response to TRAP-6 stimulation has been uniquely described previously by Pourtau et al. who tested platelet reactivity in patients with paroxysmal and persistent AF, as well as in a healthy control group [37]. They hypothesized a pre-activation of platelets in patients with AF due to prior stimulation by thrombin coming along with desensitization and reduced response to TRAP-6. This effect seems to be aggravated in patients with paroxysmal compared to persistent AF.

Interestingly, the elevated prothrombotic platelet status can be abolished by successful therapy of AF as shown by Makowski et al. and Lim et al. in small study groups [38]. Makowski et al. reported a significant decrease of platelet reactivity markers (e.g., P-selectin expression, mean platelet volume, and CD42b expression) one month after successful electrical cardioversion in 36 male patients with lone AF. P-selectin expression was additionally diminished in patients without AF recurrence documented by 24 h Holter-ECG [39]. Lim et al. analyzed expression of platelet activation markers P-selectin and PAC-1 in 57 patients undergoing catheter ablation of AF. A significant decline of investigated parameters was shown for patients with SR maintenance after 6 months compared to those with AF recurrence. These results highlight the effect of AF on platelet activation independent of comorbidities and provide interesting insights into the link between platelet activation and AF. Platelet activation markers such as P-selectin could potentially serve as surrogate parameters for AF recurrence after successful SR restoration, but further research on this topic should be conducted.

4.3. Impact of the sampling site

Most studies assessing risk of thrombosis in AF used peripheral blood samples from the cubital or femoral vein, which poorly reflect activation state of platelets in LA [3,5,6,40]. Willoughby et al. have shown a specific increase of platelet activation, especially P-selectin, in patients with AF in the LA compared to right atrium (RA) and femoral vein [41]. Furthermore, Lim et al. discovered a greater extent of platelet reactivity and thrombin generation in LA than in RA or peripheral site in acute AF and rapid stimulated atria with additional effect of AF on endothelial dysfunction and inflammation [19]. Therefore, our study complements previous results using more appropriate samples for identification of platelet activation determiners.

4.4. Morphological parameters influencing prothrombotic status

The LAA is assumed to be the most thrombogenic site in AF via fulfilling Virchow's triad [18,42]. In recent studies, the impact of LAA morphology on stroke risk has been shown [13]. A chicken wing shaped LAA is inversely related to stroke risk whereas cauliflower morphology is an independent predictor for stroke [43,44]. Furthermore, LAA function assessed by echocardiographic study has been shown to correlate significantly with stroke risk in AF patients. Measured low emptying velocities in LAA of AF patients were associated with thrombus formation and consecutive cardioembolic stroke [43,45,46]. Interestingly, this effect can be further enhanced by congestive heart failure [42]. Additional, larger LAA orifices promote a higher stroke risk [43,46]. According to these findings, our results show a possible link of morphological risk factors and triggered prothrombotic state in AF mediated by platelet activation.

4.5. Non-morphological factors attenuating platelet activation

In our study, we observed a reduced platelet reactivity to TRAP-6 in patients with heart failure. This might be due to desensitization of pre-activated platelets leading to elevated baseline platelet activation status and reduced response to TRAP-6.

The presence of abnormal platelet activation in heart failure observed in our study has been previously described [47,48]. The effect is supposedly due to the reduced blood flow and consecutive stasis, a hypercoagulable blood state, and other parameters resulting in a higher stroke prevalence [42,48]. An additional effect of heart failure therapeutics is still being discussed [48].

A difference of platelet activation status relating to gender is possible, though in our study this was not shown to be significant. Comparable to heart failure, women showed a decreased platelet reactivity to TRAP-6. Many reasons for the elevated stroke risk in female AF patients are hypothesized, but there is no data for altered platelet activation status compared to men in AF even though platelet reactivity is elevated in female patients in general [49,50].

4.6. Study limitations

Platelet function tests are useful assessments of cellular pathway of coagulation; nevertheless they are poorly standardized. However, to minimize this bias, all analysis was performed in a GCP- and CE-

certified laboratory with documented expertise in hemostaseology. Due to ethical considerations, the study is limited by a missing control group consisting of healthy donors and is of exploratory nature. We have focused on assessment of platelet activation status by means of light transmission and flow cytometry due to its good reproducibility and availability. However, there are many other possibilities to test platelet activation. Further multicenter studies should be conducted as our observations are limited by the single center population of AF patients selected for ablation.

5. Conclusion

LAA volume is associated with higher platelet activation status in patients with AF. An aggravation of this effect is shown for congestive heart failure.

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Disclosure

The authors declare that they have no conflict of interest.

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