Thrombogenic Risk in Patients With Atrial Fibrillation

Importance of Comorbid Conditions and Intracardiac Changes

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ABSTRACT

OBJECTIVES This study sought to determine the differences between the prothrombotic properties and chamber characteristics in patients with lone atrial fibrillation (AF) and those with AF and comorbidities.

BACKGROUND Thromboembolic risk is increased in patients with AF; however, whether this is due to AF per se or its comorbidities remains unclear.

METHODS A total of 87 patients undergoing ablation were prospectively recruited for the study, including 30 patients with lone AF, 30 patients with AF and comorbidities in sinus rhythm, and 27 patients with left-sided accessory pathways as controls. Blood samples were obtained from the left atrium (LA), right atrium (RA), and femoral vein (FV) after transseptal puncture. Platelet activation (P-selectin) was measured by flow cytometry. Thrombin generation (thrombin-antithrombin [TAT] complex), endothelial dysfunction (asymmetric-dimethylarginine [ADMA]), and platelet-derived inflammation (soluble CD40 ligand [sCD40L]) were measured using enzyme-linked immunosorbent assay.

RESULTS Platelet activation in the LA was significantly elevated compared to that in the FV in patients with lone AF and those with AF and comorbidities compared with that in the FV (p < 0.05 respectively). Thrombin generation was significantly elevated in the LA compared with RA in AF patients (p < 0.05). There were no significant differences in P-selectin, TAT, and sCD40L among the 3 groups. However, there was a significant stepwise increase in endothelial dysfunction measured by ADMA from controls to lone AF and then to patients with AF and comorbidities (p < 0.001 between the 2 groups).

CONCLUSIONS Patients with lone AF and those with AF and comorbidities had a greater propensity for atrial thrombogenesis than controls. Prothrombotic risk is greatest in those with comorbid conditions, in whom enhanced thrombogenesis occurs predominantly through increase in endothelial dysfunction. (J Am Coll Cardiol EP 2015;1:210–7) © 2015 by the American College of Cardiology Foundation.
The most devastating complication associated with atrial fibrillation (AF) remains thromboembolic stroke, with a 5-fold increased risk in patients with nonvalvular AF (1). Patients with AF are known to exhibit a prothrombotic state, endothelial dysfunction, and abnormal left atrial blood flow, thus fulfilling Virchow’s triad for thrombus formation (2-7). Abnormal platelet activation has been demonstrated peripherally in patients with nonvalvular AF (8,9), and peripheral blood samples have reflected endothelial dysfunction in different subsets of AF patients compared with controls (8,9).

However, there is ongoing debate as to whether these changes are due primarily to AF per se or to its associated risk factors. Several studies have suggested that in patients with AF, the prothrombotic state may be the result of concurrent comorbidities such as hypertension, diabetes, and coronary artery disease (10,11). On the other hand, other reports suggest a heightened risk of thrombosis even in patients with lone AF (8,9). Moreover, recent studies have shown that peripheral sampling may not adequately reflect intracardiac changes within the heart (4,5,12). These previous, conflicting data may be partly due to sampling from heterogeneous cohorts or sampling from peripheral versus intracardiac blood (8–11).

In this study, we examined these issues by studying both patients with lone AF and patients with AF and comorbidities, using sampling from both atrial and peripheral blood. We investigated prothrombotic properties (platelet activation, thrombin generation, endothelial dysfunction, and platelet-derived inflammation) within the left atrium (LA), right atrium (RA), and femoral vein (FV) in consecutive patients with lone AF, with AF in the setting of comorbidities, and in controls to determine the relative contribution of these factors to the thrombogenic process.

METHODS

A detailed description of methods is found in the Online Appendix.

STUDY POPULATION. Consecutive patients undergoing catheter ablation for AF at the Centre for Heart Rhythm Disorders, Royal Adelaide Hospital, were screened. Patients were studied provided they had no history of symptomatic AF in the week prior to the procedure and, by continuous monitoring for 48 h immediately prior to ablation, demonstrated no arrhythmia lasting >30 s. This rigorous screening was undertaken to minimize the impact of a recent episode of AF on the patient’s prothrombotic state. Patients were also excluded if they had an acute coronary syndrome, surgery or ablation within the preceding 3 months, chronic inflammation or infection, chronic renal or liver disease, or were taking antiplatelet therapy. All patients provided written informed consent to the study protocol, which was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, Australia.

Sixty patients undergoing catheter ablation for AF who presented in sinus rhythm were prospectively recruited (30 with lone nonvalvular AF and 30 with AF and comorbidities). The control group consisted of 27 prospectively recruited patients with left-sided accessory pathways who underwent ablation during the study period. In addition, a separate reference group of 30 age-matched subjects was recruited to control for the effect of age.

Lone AF was defined, according to previous criteria, as AF in the absence of structural heart disease, hypertension, diabetes mellitus, coronary artery disease, or stroke based on history, physical examination, chest radiography, routine biochemistry, and echocardiography (13).

STUDY PROTOCOL. The technique used for ablation of AF in our laboratory has been previously described and is further described in the Online Appendix (7,13,14). For the clinical procedure, a conventional single transseptal puncture was performed using an SLO sheath (St. Jude Medical, St. Paul, Minnesota) and a BRK-1 needle (Daig Corp., Minnetonka, Minnesota). Following transseptal puncture and before intravenous administration of unfractionated heparin, blood samples were simultaneously collected from the peripheral femoral venous sheath (FV, peripheral sample), right atrial sheath (RA) and left atrial sheath (LA). Samples from the RA and LA were collected with care using a slow withdrawal technique with the sheath positioned in the mid chamber, as previously described (4). No ablation was performed prior to completion of the study protocol. In control patients undergoing electrophysiological study and ablation of a left-sided accessory pathway, LA, RA, and FV samples were obtained immediately after transseptal puncture and before administration of heparin. Only peripheral samples were obtained for the age-matched reference group.

WHOLE-BLOOD FLOW CYTOMETRY. The surface expression of the platelet activation receptor CD62P (P-selectin) was determined by flow cytometry, using specific monoclonal antibodies (4,7). All
monoclonal antibodies were obtained from BD Biosciences (San Jose, California). The presence of platelet-expressing ligands was determined using flow cytometry (FACS_Canto machine, Becton Dickinson, Oxford, United Kingdom). Forward (size-dependent) scatter and 90° sideways (density-dependent) scatter were set at logarithmic gain, and platelets were identified on the basis of size by using the platelet immunoglobulin bead suspension. For each sample, platelets were further identified using the platelet-specific CD42b antibody (4). The control ligand (mouse immunoglobulin G2a-monoclonal antibody fluorescein isothiocyanate isotype control) was used to detect a nonspecific association and to define the threshold for activation-dependent binding. Data acquisition and analysis were performed with FACS Diva software version 4.1.2 (Becton Dickinson). The percentage of platelets expressing CD62P monoclonal antibody was defined as the fraction exhibiting specific binding (4).

ENZYME-LINKED IMMUNOSORBENT ASSAY. Blood samples were centrifuged at 2,500 g for 15 min at 4°C and stored at –80°C for batch analysis using enzyme-linked immunosorbent assay (4). Thrombin generation was assessed by measuring thrombin-antithrombin (TAT) complex (Siemens Healthcare Diagnostics, Marburg, Germany). Endothelial dysfunction was assessed by measuring levels of endogenous nitric oxide (NO) synthase inhibitor asymmetric dimethylarginine (ADMA; Immunodiagnostik, Bensheim, Germany). Platelet-derived inflammation was assessed by measuring soluble CD40 ligand (sCD40L) (R&D Systems, Minneapolis, Minnesota), according to company instructions. All assays were performed in duplicate or triplicate.

**STATISTICAL ANALYSIS.** All values are expressed as mean ± SD or as n (%) for continuous and categorical variables, respectively, unless otherwise indicated. Data were tested for normality by histogram and by using the Kolmogorov-Smirnov test and log-transformed at base e as appropriate. A constant of 1 was added to ADMA values before log transformation. Continuous variables were compared using t-1-way analysis of variance or Student t tests as appropriate. Categorical variables were compared using Fisher exact or Pearson chi-square test. For comparisons between patient groups, a linear mixed effects model was fitted to the data, with patient group fitted as a fixed effect, whereas individual patients were fitted as a random effect. To compare levels among sampling sites of individual patients within a group, a linear mixed effects model was fitted to the data within each group. In the model, sampling site was fitted as a fixed effect, whereas individual patients were fitted as a random effect. These models accounted for the samples taken from different sites from each individual patient. Subsequent Bonferroni multiple comparisons testing was performed where appropriate. Statistical significance was established at a p value of <0.05. All values were analyzed using PASW statistics 18 (version 18.0.0, SPSS, Chicago, Illinois).

**RESULTS**

**PATIENT CHARACTERISTICS.** Baseline characteristics are displayed in Table 1. Patients with left-sided accessory pathways were younger and were taking fewer antiarrhythmic agents. Patients with lone AF and those with AF and comorbidities had larger LA dimensions compared with those of control patients.

**PLATELET ACTIVATION.** There were no significant differences between levels of platelet activation measured by P-selectin in patients with lone AF, those in patients with AF and comorbidities, and those of control groups (p = 0.5 between groups).
Patients with lone AF displayed significant differences in platelet activation between the 3 sampling sites \((p = 0.014\) between sites\). Expression levels of platelet P-selectin were significantly elevated in the LA \((\text{Ln P-selectin } 2.7 \pm 0.1\% \text{ vs. } 2.5 \pm 0.1\%; p = 0.019)\) and RA \((\text{Ln P-selectin } 2.7 \pm 0.1\% \text{ vs. } 2.5 \pm 0.1\%; p = 0.026)\) compared with those in the FV \((\text{Figure 1A})\). In patients with AF and comorbidities, platelet activation in the LA was significantly elevated compared with that in the FV \((p = 0.019\) between sites; \(\text{Ln P-selectin } 2.9 \pm 0.1\% \text{ in LA vs. } 2.7 \pm 0.1\% \text{ in FV}; p = 0.036)\), and there was a trend toward increased RA levels compared with FV levels \((\text{Ln P-selectin } 2.9 \pm 0.1\% \text{ in LA vs. } 2.7 \pm 0.1\% \text{ in FV}; p = 0.08)\) \((\text{Figure 1B})\). In control patients, there were no significant differences between sites \((p = 0.1)\) \((\text{Figure 1C})\).

**THROMBIN GENERATION.** There were no significant differences in levels of TAT between patients with lone AF, those with AF and comorbidities, and controls \((p = 0.7\) between groups\). There was a trend toward increased TAT levels in the LA samples compared with the RA samples in both the patients with lone AF \((p = 0.1\) between sites: \(\text{Ln TAT } 2.8 \pm 0.1\% \text{ in LA vs. } 2.6 \pm 0.1\% \text{ in RA})\) and patients with AF and comorbidities \((p = 0.1\) between sites: \(\text{Ln TAT } 2.6 \pm 0.1\% \text{ in LA vs. } 2.4 \pm 0.1\% \text{ in RA})\). When we examined all AF patients (both lone AF and AF with comorbidities) as a single cohort, the TAT level was significantly higher in the LA than in the RA \((p = 0.037\) between sites: \(\text{Ln TAT } 2.7 \pm 0.1\% \text{ in LA vs. } 2.5 \pm 0.1\% \text{ in RA}; p = 0.033)\), whereas there were no significant site differences in the control group \((p = 0.2)\) \((\text{Figure 2})\).

**ENDOTHELIAL DYSFUNCTION.** Comparing between groups, there was a significant stepwise increase in ADMA levels of all 3 sites from controls to patients with lone AF and then patients with AF and comorbidities \((p < 0.001\) between groups; mean \(\text{Ln ADMA } 0.29 \pm 0.01 \mu M/l\) in controls vs. \(0.34 \pm 0.01 \mu M/l\) in patients with lone AF, \(p = 0.015\); \(\text{Ln ADMA } 0.34 \pm 0.01 \mu M/l\) in lone AF vs. \(0.40 \pm 0.01 \mu M/liter\) in patients with AF and comorbidities, \(p = 0.031)\) \((\text{Figure 3})\). These differences remained significant \((p < 0.01\) between groups) after adjustment for age.

In addition, to control for the effect of age as a possible cause for the increase in ADMA levels, we studied a reference group of 30 healthy age-matched subjects without cardiovascular comorbidities \((n = 30, 50\% \text{ male})\). The average age of the reference group was 54.5 \pm 6.6 years of age \((p = 0.5 \text{ compared with AF patients})\), and average body mass index was 26.6 \pm 4.8 kg/m\(^2\) \((p = 0.2 \text{ compared with the other 3 groups})\). Peripheral samples were obtained from these patients and compared with the peripheral levels of the other 3 groups. There were significant differences in peripheral ADMA levels between groups \((p < 0.001)\): peripheral ADMA levels were significantly elevated in patients with lone AF and in patients with AF and comorbidities compared with those in the
There were no significant differences in sCD40L levels between the LA, RA, and FV sites in patients with lone AF (Ln sCD40L: 5.1 ± 0.1 pg/ml in LA vs. 5.2 ± 0.1 pg/ml in RA vs. 5.1 ± 0.1 pg/ml in FV, p = 0.5), AF patients with comorbidities (Ln sCD40L: 5.1 ± 0.1 pg/ml in LA vs. 5.1 ± 0.1 pg/ml in RA vs. 5.0 ± 0.1 pg/ml in FV, p = 0.5), and controls (Ln sCD40L: 4.9 ± 0.1 pg/ml in LA vs. 5.1 ± 0.2 pg/ml in RA vs. 5.1 ± 0.1 pg/ml in FV, p = 0.3).

At 6-month follow-up, none of the patients in the study sustained a stroke or transient ischemic attack.

**DISCUSSION**

This study presents new information on the contribution of AF and its associated comorbidities to prothrombotic risk and the chamber-specific effects of these prothrombotic markers within the atria and peripheral circulation. The main findings of the study were: 1) patients with lone AF and those with AF and comorbidities had a greater propensity for atrial thrombogenesis than controls; and 2) a significant stepwise increase in endothelial dysfunction was observed from controls to patients with lone AF and then to patients with AF and comorbidities. These findings suggest that comorbid conditions enhance thrombogenesis predominantly through increase in endothelial dysfunction, and both AF per se and its associated comorbidities contribute to prothrombotic risk.

**PLATELET ACTIVATION AND PLATELET-DERIVED INFLAMMATION IN AF PATIENTS.** Platelet expression of P-selectin is a commonly used marker of platelet activation and is associated with presence of LA thrombus or embolic events in patients with AF (4,12,15). The present study, which assessed platelet activation remote from an arrhythmia episode, did not find significant differences between the groups. This is consistent with a previous study that reported no significant differences in abnormal platelet activation between AF patients and controls with underlying cardiovascular comorbidities (10). On the other hand, several studies have shown that platelet activation is increased by the onset of AF in a time-dependent manner (4,5,16). These findings suggest that the arrhythmia episode or AF duration, rather than the baseline state, may have a larger impact on platelet activation. Similarly, although the onset of AF has been associated with increased sCD40L expression, a marker of platelet-derived inflammation (4), in this study, sCD40L levels were not significantly different between the groups at a state remote from
arrhythmia. This also suggests that platelet-derived inflammation may be related more to the arrhythmia episode, analogous to previous findings that higher CRP levels were observed in patients with AF present within 24 h prior to sample collection compared with sinus rhythm (17).

**ENDOTHELIAL DYSFUNCTION IN PATIENTS WITH AF AND COMORBID CONDITIONS.** Endothelial dysfunction is independently associated with clinical stroke risk factors in patients with nonvalvular AF and is predictive of subsequent vascular events (4). ADMA is an endogenous inhibitor of endothelial NO synthase and is known to result in endothelial dysfunction in human studies (18). Clinically, ADMA is raised in numerous cardiovascular conditions and predicts mortality in cardiovascular patients (4).

The present study demonstrated a stepwise increase in ADMA levels from controls to patients with lone AF and then to patients with AF and comorbidities. This indicates that both AF per se and its associated comorbidities contribute to endothelial dysfunction and prothrombotic risk. Atrial substrate abnormalities and structural damage have been demonstrated in patients with lone AF and are posited as essential contributors to the “second factor” that promotes development and progression of AF (13,19). Local endothelial changes such as patchy fibrosis and increased inflammatory infiltrates have been demonstrated in AF patients from surgical and autopsy series (20,21). Furthermore, electrophysiological studies reveal structural atrial abnormalities characterized by loss of myocardial voltage and conduction slowing in patients with lone AF (13). However, there is also concurrent evidence that AF induces endothelial dysfunction (4–6,22). In human studies, the acute onset of AF is associated with elevated ADMA levels (4), whereas animal models of AF result in increased ADMA levels and decreased NO bioavailability (6,22). Reduced NO levels are correlated with increased prothrombotic proteins and platelet activation, thus enhancing prothrombotic risk (15,22). The present study findings indicate that comorbid conditions enhance thrombogenesis predominantly through increase in endothelial dysfunction. This corroborates previous studies that have demonstrated endothelial dysfunction with cardiovascular diseases such as hypertension, diabetes mellitus, and coronary artery disease (4,23).

**INCREASED ATRIAL THROMBOGENICITY IN PATIENTS WITH AF.** In the present study of patients with AF, abnormal platelet activation was more pronounced in the atria than in the periphery, and thrombin generation was more pronounced in the LA than in the RA.

Why is thrombogenicity more pronounced in the atria and specifically in the LA? The mechanisms are incompletely understood, but there are several possible explanations in which abnormal blood constituents, abnormal blood flow, and abnormal vessel wall (components of Virchow’s triad) may interact and promote thrombogenesis within the atria. First, abnormal blood flow and blood stasis occur due to a dilated LA in patients with AF (3). The phenomenon of spontaneous echo contrast is independently related to hematologic parameters such as fibrinogen and LA dilation (24). Furthermore, LA size positively correlates with markers of endothelial dysfunction and coagulation factors in AF patients (8).

Second, there is evidence of local endothelial dysfunction and vessel wall damage in the atria. Abnormal atrial histology has been demonstrated in patients with paroxysmal lone AF (20). In patients with mitral valve disease, immunoreactive von Willebrand factor (vWF), a marker of endothelial dysfunction, was more frequently observed in the endocardial LA appendage than in the RA appendage, which correlated with the degree of platelet adhesion and thrombus formation in the endocardium (21). AF is associated with a decrease in endocardial NO synthase expression from animal models (15,22). Elevated levels of ADMA, as indicated in the present study, an endogenous inhibitor of endothelial NO synthase, further result in decreased NO bioavailability and
endothelial dysfunction through loss of its antithrombogenic properties \((4,12,18)\). The decrease in NO bioavailability in AF is associated with increased platelet P-selectin expression and expression of prothrombotic proteins such as plasminogen activator inhibitor-1 from animal models (third component of Virchow’s triad) \((15,22)\). Furthermore, increased oxygen tension in the LA may be associated with increased superoxide levels, further reduction in NO bioavailability, and abnormal platelet activation \((12,22)\). Finally, hemodynamic differences between the LA and the RA may result in more marked structural and endothelial changes in the LA \((19)\).

**CLINICAL IMPLICATIONS.** Both AF per se and its comorbid conditions enhance thrombotic risk through endothelial dysfunction. Management of cardiovascular comorbid conditions and strategies to treat AF should therefore focus on therapies to improve endothelial function. This study also highlights the importance of strict management of concurrent cardiovascular comorbidities in the AF patient, as the prothrombotic risk seems to be compounded \((25)\). Third, as these changes appear to be localized, future research, assessment of disease progress, and response to therapy in AF should emphasize the local effects in the atria \((4,12)\).

**STUDY LIMITATIONS.** Both patients with paroxysmal AF and those with persistent AF were included in this study. It is possible that duration of AF affects the degree of platelet activation; however, we attempted to control for these effects by including only patients who had no arrhythmia for 48 h prior to sampling. In this study cohort, there were no significant differences between biomarker levels in paroxysmal AF patients and those in persistent AF patients (Ln P-selectin \(p = 0.9\); Ln TAT \(p = 0.3\); Ln ADMA \(p = 0.1\); Ln sCD40L \(p = 0.6\)). Second, the study was limited in subject numbers because of the strict definition of lone AF, in addition to the exclusion criteria of patients with recent onset of arrhythmia. Third, history of smoking might have had an effect on endothelial function in patients, although there were no significant differences in proportions of patients with a history of smoking between groups. Fourth, despite stopping warfarin administration 7 days prior to the procedure and excluding patients on antiplatelet agents, it is not possible to rule out the effect of warfarin or other concomitant medication on platelet activation characteristics. Nevertheless, regional differences within each individual patient should not be affected. Fifth, although this study demonstrated increased prothrombotic risk in patients with lone AF and in those with AF and comorbidities, whether these findings translate to clinical thromboembolic events warrants further investigation.

**CONCLUSIONS**

Patients with lone AF and those with AF and comorbidities had a greater propensity for atrial thrombogenesis than controls. Both AF per se and its associated comorbidities contribute to prothrombotic risk; however, the prothrombotic risk is greatest in those with comorbid conditions, in whom enhanced thrombogenesis occurs predominantly through increase in endothelial dysfunction.

**REFERENCES**

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KEY WORDS atrial fibrillation, endothelial dysfunction, left atrium, stroke prevention, thrombotic risk

APPENDIX For supplemental material, please see the online version of this article.