Atrial Fibrillation-Associated Remodeling Does Not Promote Atrial Thrombus Formation in Canine Models

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Background—The most important complication of atrial fibrillation (AF) is thromboembolic stroke. Although AF-related remodeling is considered important in atrial thrombogenesis, its role never has been directly tested. This study assessed effects of AF-related remodeling on the atrial thrombogenic milieu by using radiofrequency ablation (RFA) to create a quantifiable prothrombotic nidus.

Methods and Results—We studied normal control dogs (control, n=16) and 3 canine AF-models: (1) atrial tachycardia remodeling (ATR; n=16) induced by atrial tachypacing (400 bpm for 1 week, with atrioventricular block and ventricular pacing at 80 bpm); (2) congestive heart failure (CHF; n=14) attributable to ventricular tachypacing (240 bpm for 2 weeks); and (3) chronic AF (CAF; n=8) induced by atrial tachypacing (35±3 days) without atrioventricular block. CAF dogs had AF for 13±1 days until euthanization. After remodeling was established, RFA lesions were created in both atria. Half the ATR and CHF dogs were subjected to atrial tachypacing during 7-day post-RFA follow-up. Electrophysiological and echocardiographic studies were performed before RFA and 7 days after RFA, and then hearts were removed and atrial thrombus were quantified by histomorphometry. Burst-pacing–induced AF duration was significantly greater in ATR, CHF, and CAF groups versus control group. The atrial effective refractory period shortened in ATR and CAF groups. Left atrial diameter was significantly larger with CHF, but not with ATR. Neither total thrombus volume nor thrombus volume per lesion differed significantly among groups.

Conclusions—None of the AF substrates tested, including sustained atrial tachycardia/AF itself, enhanced post-RFA atrial thrombus formation. Indices of electrical and structural remodeling did not predict post-RFA thrombogenic potential. Contrary to widely held but previously untested notions, we were unable to demonstrate prothrombotic effects of AF-related remodeling. (Circ Arrhythm Electrophysiol. 2012;5:1168-1175.)

Key Words: atrial fibrillation ■ remodeling ■ thrombus

Atrial fibrillation (AF) is a common and problematic sustained arrhythmia.1-3 The most important AF-associated clinical complication is thromboembolic stroke.4 AF is the single largest risk factor for stroke in the elderly,4 and it is associated with particularly severe strokes.5 Despite the significance of the thromboembolic risk of AF, relatively little is known about the mechanistic factors governing AF-related thromboembolism.6

Loss of atrial contraction and blood stasis attributable to high-frequency atrial activation during AF is believed to be important in atrial thrombus formation. However, there are some puzzling aspects of the epidemiology of AF-related thromboembolism. There are no clear differences in thromboembolic risk between patients with paroxysmal versus persistent AF;7,8 and sinus rhythm (SR) maintenance has not been shown to prevent AF-related stroke.9,10 There clearly is a need for a better understanding of the fundamental determinants of AF-associated thromboembolism.

Atrial stasis in AF is not solely attributable to the arrhythmia per se; it also is attributable to atrial mechanical abnormalities induced by AF-induced remodeling.11-13 There is indirect evidence that atrial remodeling may contribute to thromboembolic risk. Atrial tachycardia (AT) remodeling (ATR) downregulates atrial...
endocardial nitric oxide synthase and nitric oxide production, with consequent upregulation of prothrombotic plasminogen activator inhibitor-1, suggesting increased thrombogenicity. 

Gene microarray analysis showed upregulation of procoagulant factor genes in AF patients, and thrombosis-related genes are upregulated in a canine model of congestive heart failure (CHF)-induced atrial structural remodeling. Despite the enormous clinical importance of AF-associated thromboembolism, there are no published direct experimental assessments of the influence of putative AF-related thrombosis-promoting conditions on atrial thrombus formation. This study aimed to establish whether AF-related remodeling enhances the risk of atrial thrombus formation. Specific objectives included determining whether AF-promoting remodeling (ATR-induced or CHF-induced) favors the formation of atrial thrombi, and determining whether persistent rapid atrial activation (AT or AF) interacts with previous atrial remodeling to promote thrombus formation. Because of the low occurrence rate of spontaneous thrombus formation, we used radiofrequency ablation (RFA) to produce thrombus formation to assess the prothrombotic tendencies of various AF-related paradigms.

**Methods**

Detailed Methods are available in the online-only Data Supplement.

**Experimental Groups**

Animal handling procedures were approved by the local Animal Research Ethics Committee. Fifty-four mongrel dogs (weight, 20.7–38.2 kg) were studied in the following groups (Figure 1): (1) normal control dogs kept in SR for 7 days before RFA and the 7-day post-RFA follow-up period (control group); (2) dogs with ATR induced by 7-day atrial tachypacing (ATR group); (3) dogs with CHF induced by ventricular tachypacing applied for a 14-day pre-RFA preparation and 7-day post-RFA follow-up period (CHF group); and (4) dogs with chronic AF (CAF) induced by long-term atrial tachypacing (CAF group). After the preparation periods, 4 RFA lesions were created in each atrium. Dogs were subjected to electrophysiological study (EPS) 7 days after RFA, followed by euthanasia, cardiac excision, and thrombus quantification. In ATR and CHF groups, half the dogs were subjected to atrial tachypacing during the 7-day post-RFA follow-up period (ATR-AT and CHF-AT subgroups), whereas the others were left in sinus node-driven atrial rhythm during 7-day post-RFA follow-up (ATR-SR and CHF-SR subgroups).

**Echocardiography**

Transthoracic echocardiography was performed under sedation (Atravet 0.07 mg/kg; buprenorphine 0.009 mg/kg, intramuscularly) before RFA and at terminal study. All recordings were performed in SR except for the terminal study in CAF dogs, in which echocardiography was performed in AF to avoid direct-current cardioversion-induced dislodging of the atrial thrombus. For details of echocardiographic measurements, see online-only Data Supplement. The average of 3 to 6 cardiac cycles was used for each measurement, with the operator blinded to treatment assignment.

**EPS**

Closed-chest EPS was performed before RFA and at terminal study for control, ATR, and CHF groups. During EPS, tachypacing makers were deactivated. In CAF dogs, AF was direct-current-cardioverted for the pre-RFA EPS, but cardioversion was not performed at terminal study to avoid thrombus dislodgment. EPS was performed in SR (and therefore was not performed at the terminal study in CAF dogs). Effective refractory period (longest premature coupling interval [S1S2] failing to capture) was measured in right atrial appendage (2xthreshold-current, 2-ms pulses, 3 determinations) at basic cycle lengths of 150, 200, 250, 300, and 360 ms with 10 basic stimuli (S1s), followed by premature extrastimuli (S2s) with 5-ms decrements. AF was induced by 1-s to 10-s burst pacing; 10 Hz; 4x threshold current. Mean AF duration was based on 10 AF inductions for AF <20 min and 5 AF inductions for AF 20 to 30 min. Prolonged AF (>30 min) was terminated by cardioversion. If prolonged AF was induced twice, then no further AF induction was performed.

**RFA**

RFA was performed during SR in control, ATR-SR, and CHF-SR subgroups, during atrial tachypacing in ATR-AT and CHF-AT subgroups, and during AF in the CAF group. Dogs were anesthetized (morphine [2 mg/kg, subcutaneous] and α-chloralose [120 mg/kg, intravenous load, 29.25 mg/kg per hour]) and ventilated mechanically. A nonirrigated 7-F quadripolar radiofrequency catheter (Biosense Webster) with a 4-mm distal electrode tip was positioned under fluoroscopic and intracardiac echocardiographic guidance. Access to the left atrium (LA) was obtained by transeptal puncture. RFA lesions were produced at 50 W, with

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**Figure 1.** Schematic of groups and interventions. AF indicates atrial fibrillation; AT, atrial tachycardia; ATR, atrial tachycardia remodeling; CAF, chronic atrial fibrillation; CHF, congestive heart failure; CTL, control; RFA, radiofrequency ablation; SR, sinus rhythm; and VTP, ventricular tachypacing.
target temperature of 70°C, for 60 seconds. Maximum impedance value, temperature, and power output averaged 128.1±1.7 Ω, 68.5±0.2°C, and 13.4±0.6 W, respectively. Sheaths were initially flushed with heparinized saline (1000 IU/L); no other anticoagulation was used during the protocol.

Blood Samples
Citrated blood samples from a femoral vein were obtained at the terminal study. Plasma was removed and stored at -80°C. The concentrations of a coagulation activation marker, thrombin–antithrombin complexes, and an inflammation marker, C-reactive protein, were analyzed by enzyme-linked immunosorbent assay.

Histology and Histochemistry
After animal euthanasia, hearts were removed, fixed in 10% formalin, and subjected to histological processing. Maximal length and width of ablation lesions were measured. Paraffin-embedded specimens were serially sectioned at a thickness of 6 µm. Thrombi were defined and quantified as described previously. Personnel analyzing lesion morphometry were blinded to experimental group. Mean thrombus volume and total thrombus volume were calculated for each dog.

Data Analysis
Continuous variables are expressed as mean±SEM for normally distributed variables (Shapiro-Wilk test) or median and first and third quartile values (Q1-Q3)/minimum-maximum values for non-normal variables. Multiple group comparisons were obtained with 1-way ANOVA with Bonferroni-adjusted t tests (for normally distributed data) or Kruskal-Wallis test with Dunn multiple comparison test (for non-normally distributed data) for nonrepeated measures (main effect factor dog group). Repeated-measures analyses were performed for normally distributed data with 2-way ANOVA and Bonferroni-adjusted t tests. Two-tailed P<0.05 indicated statistical significance. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
Electrophysiologic Characteristics
The electrophysiologic characteristics of each group of dogs after the pre-RFA preparation period are shown in Figure 2. Data were not different between SR and AT subgroups in ATR and CHF dogs before RFA, and therefore are expressed per model group of dogs before RFA and at terminal study are shown in online-only Data Supplement Table I. Data were not significantly different between SR and AT subgroups of ATR and CHF dogs before RFA. In CHF, left ventricular diastolic and systolic volumes were larger, and left ventricular ejection fraction was lower compared with controls both before RFA and at terminal study. Left ventricular ejection fraction was slightly but significantly lower, without significant left ventricular dilatation, in CAF. LA size was substantially larger and LA fractional area change was lower in CHF vs control groups. Late atrial-dependent ventricular filling (A)-wave velocity was lower and early/late ventricular filling velocity (E/A) ratio was higher in ATR group versus control group (no A-wave was present after RFA in the CAF group because echocardiograms were obtained in AF; online-only Data Supplement Table I). Post-RFA SR reversed LA remodeling in ATR-SR, with statistical significance between pre-RFA and terminal studies observed in LA systolic and diastolic areas, A-wave velocity, and E/A ratio (online-only Data Supplement Table I). In CHF-AT, post-RFA AT further increased LA diastolic size (diameter and area) and decreased LA contractility (fractional shortening and fractional area change) significantly, whereas A-wave velocity decreased and E/A ratio increased significantly (online-only Data Supplement Table I). No images compatible with thrombus per se were obtained.

Plasma Thrombin–Antithrombin Complexes and C-Reactive Protein Concentrations
Plasma thrombin–antithrombin complexes and C-reactive protein concentrations for each group of dogs at the terminal
study are shown in Figure 4. Full subgroup-based data are shown in online-only Data Supplement Figure II. Plasma thrombin–antithrombin and C-reactive protein concentrations were not different among groups.

Ablation Lesion and Thrombus Sizes

The characteristics of RFA lesions and thrombi are presented in the Table. Subgroup-based lesion characteristics in each region are shown in online-only Data Supplement Table II. The number of successfully performed ablation lesions and mean lesion area, depth, and volume did not differ among groups. More than 70% of lesions were associated with thrombus and there were no significant differences compared with controls in the prevalence or number of thrombi among groups. Representative examples of thrombus histopathology from a control and a CAF dog are shown in Figure 5A and 5B, respectively. Thrombi were clear and amenable to histomorphometric quantification; no systematic qualitative differences in thrombus histomorphology were seen. Mean thrombus size data are shown in Figure 5C. Representative examples of histopathology and mean thrombus size data from all subgroups are shown in online-only Data Supplement Figure III. There were no significant differences in total thrombus volume per dog among groups (Figure 5C). Similarly,

<table>
<thead>
<tr>
<th>Table.</th>
<th>Properties of Ablation Lesions and Atrial Thrombi</th>
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<tr>
<td></td>
<td>Experimental Group</td>
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<tr>
<td></td>
<td>Control (n=16) ATR (n=16) CHF (n=14) CAF (n=8)</td>
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<tr>
<td>N of ablation lesions per dog</td>
<td>6.9±0.3</td>
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<tr>
<td>Ablation lesion area, mm²</td>
<td>53.1±3.5</td>
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<tr>
<td>Ablation lesion depth, mm</td>
<td>5.2±0.2</td>
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<tr>
<td>Ablation lesion volume, mm³</td>
<td>205.2±17.8</td>
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<tr>
<td>N of thrombi per dog</td>
<td>5.4±0.4</td>
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<tr>
<td>Presence of thrombus, %</td>
<td>80±5</td>
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<tr>
<td>Mean thrombus volume in both atria, mm³</td>
<td>20.8±3.4</td>
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<tr>
<td>Mean thrombus volume in left atria, mm³</td>
<td>8.2±1.5</td>
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<tr>
<td>Mean thrombus volume in right atria, mm³</td>
<td>30.1±5.4</td>
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<tr>
<td>Total thrombus volume in both atria, mm³</td>
<td>140.5±21.3</td>
</tr>
<tr>
<td>Total thrombus volume in left atria, mm³</td>
<td>22.8±5.3</td>
</tr>
<tr>
<td>Total thrombus volume in right atria, mm³</td>
<td>117.7±21.5</td>
</tr>
<tr>
<td>Thrombus volume normalized to ablation lesion area in both atria, mm³/mm²</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Thrombus volume normalized to ablation lesion volume in both atria</td>
<td>0.2±0.1</td>
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ATR indicates atrial tachycardia remodeling; CAF, chronic atrial fibrillation; and CHF, congestive heart failure.

*There were no statistically significant differences for any groups vs control group for any of these variables studied.
there were no significant differences when thrombus volume was normalized to number of lesions per dog or lesion area (Table). In addition, there were no specific atrial regions in which thrombus size differed (online-only Data Supplement Table II). Thrombi formed only in RFA sites.

Discussion

Atrial thromboembolism is the most significant complication of AF. Based primarily on inferences from clinical observations, a variety of mechanisms have been suggested to contribute to AF-related thromboembolism. However, there are virtually no experimental data available about the basic factors governing atrial thrombus formation in AF models. Here, we examined the effects of a variety of AF-associated paradigms on atrial thrombus occurrence and volume. We found that, contrary to expectations based on suggestions in the literature, none of these paradigms promoted thrombus formation in our model.

Determinants of Atrial Thrombogenesis in AF

Atrial thrombi causing cardioembolic stroke are typically red cell–rich “red thrombi” typical of low-flow, stasis-associated thrombi, like those occurring in leg veins.22,23 Echocardiographic indices of LA stasis are useful predictors of thromboembolic risk in AF and are often relied on as key indicators of the safety of direct-current cardioversion.24 Atrial stasis in AF has been suggested to be not only attributable to the rapid and disorganized rhythm per se but also attributable to atrial contractile abnormalities induced by the atrial remodeling resulting from AF.11-13 AF-related remodeling causes atrial hypocontractility via multiple factors, including L-type Ca2+ current reduction, impaired subcellular Ca2+ signal transmission, and altered myofilament function associated with abnormal myosin and myosin-associated protein phosphorylation.11-13

In addition to blood stasis, there is evidence that the other key factors of the Virchow triangle, endothelial thromboresistance and blood coagulability, are altered in AF and AF-related remodeling, and that these changes are important in atrial thromboembolism.25 An association between increased C-reactive protein concentrations and AF occurrence suggests a possible inflammatory component.26,27 C-reactive protein associates with echocardiographic indices of atrial stasis, supporting an “inflammatory hypothesis” related to thrombogenic risk in AF.27 These inflammatory processes, together with increased expressions of growth factors, such as tissue factor, vascular endothelial growth factor, and the vascular endothelial growth factor receptor sFlt-1, may contribute to endothelial thrombogenic dysfunction.28-30 Upregulation of von Willebrand factor is compatible with AF-related thrombogenic endothelial dysfunction.29 In atrially tachypaced pigs, nitric oxide synthase expression and nitric oxide production are downregulated, with associated upregulation of prothrombotic plasminogen activator inhibitor-1 in the LA endothelium.14 Thrombomodulin and tissue factor pathway inhibitor are downregulated in the LA endothelium of atrially tachypaced rats.30 CHF is a risk factor for AF-related thromboembolism.31 The basis for this association is unknown, but CHF causes abnormal clotting properties, even in SR patients,32 and promotes atrial dilation and contraction disturbances.

RFA damages endocardial endothelium and causes local inflammation, producing local thrombus formation.17 Thromboembolic complications occur in approximately 0.8% of patients undergoing RFA, with the prevalence closer to 2% for RFA in the left heart.33
Atrial Thrombogenic Risk in AF

Novel Aspects and Potential Significance

There are no published experimental studies of the effect of atrial remodeling on atrial thrombus formation. Although previous studies point to an increased thrombotic risk based on changes in biomarkers of coagulation and inflammation,\(^\text{26-30}\) direct prothrombotic effects have never been assessed. We designed this study to investigate the effects of AF-related remodeling on atrial thrombotic risk, considering many factors proposed in the literature to promote thrombogenesis in AF, such as ATR, CHF, atrial dilation, and AF itself. The electrophysiological changes in each subgroup are consistent with results of previous studies in the respective models.\(^\text{21}\) We built into the design an analysis of both pre-RFA and post-RFA remodeling with the use of rhythm paradigms that incorporated SR or atrial tachypacing in a controlled way after the application of RFA. To our great surprise, none of the interventions we analyzed promoted atrial thrombus formation. Our observations challenge the widely believed notion that AF-associated remodeling directly promotes atrial thrombus formation.

Potential thrombogenic properties of the remodeled atrial milieu have important therapeutic implications. If, for example, atrial inflammation, oxidant stress, or deficient endothelial thromboprotection attributable to AF contributes to atrial thrombosis, then therapies directed against these changes and the underlying remodeling should protect against AF-related stroke and other complications. It is conceivable that such protection could be achieved with less associated risk than systemic anticoagulation, offering supplemental benefit to oral anticoagulants or a component of alternative therapy for patients at high risk for bleeding. Our results argue that additional experimental work is needed to directly establish the role, if any, of various atrial remodeling–related factors in AF-associated thromboembolism.

Considerations of the Model

We elected to use RFA to promote atrial thrombus formation. We reasoned that if any of the remodeling paradigms that we studied promote atrial thrombogenesis, then they would increase thrombus size and alter the thrombotic biomarkers that we studied. Our studies exclude such a prothrombotic state. Ideally, we would have liked to study spontaneous thrombus formation, but we anticipated a low prevalence of spontaneous thrombus formation, an expectation that was borne out by the lack of thrombi in atrial areas other than those exposed to RFA. Even in patients at highest risk, with CHADS\(_2\) scores >5, annual thromboembolic rates of >6% per year have been reported,\(^\text{34}\) although much higher rates of thromboembolic events have been reported in patients who were not anticoagulated after hospital discharge.\(^\text{35}\) In patients with subtherapeutic anticoagulation before AF cardioversion, the prevalence of echocardiographically detectable thrombi is <10%.\(^\text{36}\) Thus, spontaneous atrial thrombus formation may be difficult to observe in animal models that mimic human AF.

Our findings raise some interesting questions about the thromboembolic risk of RFA in AF ablation or in patients with AF. If AF-related remodeling causes a prothrombotic state, then RFA-related thromboembolic events would be more likely in patients undergoing RFA in the presence of the arrhythmia. Our results suggest that this may not be the case, a notion that should be amenable to testing in databases and clinical trials.

Like any experimental study, extrapolation of our findings to humans should be very cautious. Our models do not perfectly mimic any clinical situation, and the coagulation system of dogs may be different from that of humans. Our study was relatively short-term. Dogs were monitored for 7 days after RFA, and the longest remodeling interval (in CAF dogs) was 5 weeks. We elected to use relatively short-term models for practical reasons (limited dog-housing capacity), and because the previously published indirect experimental evidence for an effect of remodeling on AF-related thromboembolic risk derives from such short-term studies.\(^\text{11-14,16}\) Although data to indicate that longer periods of AF-related remodeling might influence atrial thrombogenesis are lacking, this possibility cannot be excluded and should be assessed in future work. Nevertheless, because thromboembolism is the most important complication of AF and its underlying mechanistic determinants are incompletely understood, we feel that the present study is an important first step toward further critical experimental testing of widely held notions about the factors governing AF-related thrombus formation.

Other Possible Limitations

First, although no detectable symptomatic stroke was observed in this study, brain imaging analyses (eg, by magnetic resonance imaging or computed tomography) were not performed; therefore, the possibility of silent cerebral infarctions could not be excluded. Second, although excision of the heart was performed carefully, because transesophageal echocardiographic studies were not performed, undetected dislodgement of atrial thrombi conceivably could have occurred. Future work addressing both of these concerns would be of interest. Third, although the sheaths were flushed with heparinized saline, no other anticoagulation was used during the protocol. We cannot exclude the possibility that differences among groups would be evident only in anticoagulated models. Fourth, because of the relatively small numbers of animals in each group, we cannot exclude small differences among groups that escaped detection.

Conclusions

Atrial thrombus formation is not affected by a wide range of AF remodeling paradigms in dogs. These observations challenge the notion that AF-related remodeling per se directly produces a prothrombotic state. Further study in experimental and clinical models is needed to understand more precisely the basic mechanisms underlying AF-associated thromboembolism, the most important adverse clinical consequence of the arrhythmia.

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Disclosures
None.

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Is the hypercoagulable state in atrial fibrillation mediated by vascular en-
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associated with chronic atrial fibrillation and underlying valvular heart
Thromboembolic complications, particularly stroke, are the most important adverse clinical consequences of atrial fibrillation (AF). However, little is known about the basic mechanisms underlying AF-specific thromboembolism. Indirect evidence suggests that the atrial remodeling associated with AF promotes atrial thrombus formation, thereby contributing to thromboembolic risk; however, this idea never has been tested directly. If atrial remodeling is thrombogenic, then antiremodeling approaches would have the capacity to help in preventing AF-related stroke and might be useful additions to our therapeutic armamentarium. Here, we studied atrial thrombus formation in a range of experimental paradigms of AF-associated remodeling: atrial tachycardia remodeling, heart failure–associated remodeling, and prolonged AF. We used radiofrequency ablation to create a nidus for thrombus formation. Hearts were excised 1 week after ablation and thrombus size was analyzed with quantitative histomorphometry. Ablation lesions were created in both atria in control dogs and in remodeled animals, with subgroups subjected to continued atrial tachycardia (to mimic AF) during the 7-day follow-up period or left in sinus rhythm. Atrial remodeling significantly enhanced AF susceptibility and caused the expected structural remodeling. Nevertheless, none of the remodeling scenarios studied significantly altered atrial thrombus area or volume. We conclude that atrial remodeling does not affect atrial thrombogenesis for any of the wide range of paradigms that we studied. More work in experimental models is needed to define the fundamental determinants of AF-related thrombus formation to improve our ability to prevent AF-related stroke.
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SUPPLEMENTAL MATERIALS

Atrial Fibrillation-Associated Remodeling and Atrial Thrombogenic Risk in Canine Models

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Supplemental Methods

Experimental Groups

Animal-handling procedures were approved by the local Animal Research Ethics Committee and conform with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Publication No. 85-23, revised 1996). Fifty-four mongrel dogs (weight, 20.7-38.2 kg) were studied in the following groups (Figure 1): (1) normal control (CTL) dogs, which were kept in sinus rhythm (SR) for 7 days pre-RFA and the 7-day post-RFA follow-up period (CTL group); (2) dogs with ATR induced by 7-day atrial-tachypacing for the pre-RFA preparation period (ATR group); (3) dogs with CHF induced by ventricular-tachypacing applied for a 14-day pre-RFA preparation and 7-day post-RFA follow-up period (CHF group); (4) dogs with chronic AF (CAF) induced by long-term atrial-tachypacing for the preparation period, and kept in AF thereafter (CAF group). After the preparation periods, RFA lesions were created in both atria, 4 in right (RA) and 4 in left (LA) atrium, to stimulate thrombus formation. Dogs were subjected to
electrophysiological study 7 days post-RFA, followed by euthanasia, cardiac excision and then atrial thrombus quantification. In ATR and CHF groups, half the dogs were subjected to atrial-tachypacing during the 7-day post-RFA follow-up period (ATR-AT and CHF-AT subgroups), while the other dogs were left in SR during the 7-day post-RFA follow-up period (ATR-SR and CHF-SR subgroups).

**Animal Models**

Atrial-tachypacing and ventricular-tachypacing were performed as previously described.\(^1\)\(^-\)\(^3\) For instrumentation, the dogs were anesthetized with ketamine (5.3 mg/kg, i.v.), diazepam (0.25 mg/kg, i.v.), and isoflurane (1.5%). In ATR dogs, bipolar pacing-leads were inserted into the right ventricular apex and RA appendage and connected to subcutaneous pacemakers (Vitatron, Minneapolis, MN). Atrioventricular block was created by His-bundle RFA. The right ventricular demand-pacemaker was programmed to 80 bpm. After 24-h post-operative recovery, 7-day atrial-tachypacing at 400 bpm was initiated. Atrial RFA was performed on day 7. In CHF dogs, a bipolar pacing-lead was inserted into the right ventricular apex under fluoroscopic guidance, and connected to a pacemaker. After 24-h post-operative recovery, 14-day ventricular-tachypacing at 240 bpm was initiated. RFA was performed on day 14. In CAF dogs, a bipolar pacing-lead was inserted into the RA appendage and connected to a pacemaker. Atrioventricular block was not created. Instead, oral digoxin (0.25 mg/day) was administered throughout the study period to control ventricular response. After 24-h post-operative recovery, atrial-tachypacing at 400 bpm was initiated. The electrocardiogram was then verified at least twice a week. If AF occurred, the electrocardiogram was monitored more frequently. Atrial-tachypacing was continued for 35±3 days until the dogs had CAF (at least >24 hours). AF was then maintained until the day of RFA.
(5±1 days after the development of CAF). In all the groups of dogs, a bipolar pacing-lead was inserted into the RA appendage for serial closed chest electrophysiological studies.

**Echocardiography**

Transthoracic echocardiography was performed at two time points, before RFA and at terminal study. The recording was performed under sedation (with Atravet 0.07 mg/kg and buprenorphine 0.009 mg/kg IM) using an M3S probe (2.0-4.3 Megaherz) and a Vivid 7 Dimension system (GE Healthcare Ultrasound, Horten, Norway). All recordings were performed in sinus rhythm except for the terminal study in CAF-dogs, in which echocardiography was performed in AF to avoid direct-current cardioversion with possible dislodgement of atrial thrombus. To study LA emptying and contractile function, an apical 4-chamber view was obtained, and LA areas in both cardiac end-systole (defined as the largest) and end-diastole (defined as the smallest) were measured. An M-mode echocardiogram was obtained at the aortic-valve level in the parasternal long axis view to measure LA diameters. Atrial fractional shortening was calculated as (systolic-value minus diastolic-value)/systolic-value×100% both for LA areas and diameters. Pulse-wave Doppler was used to study transmitral flow in the apical 4-chamber view. Peak velocity in early filling E-wave, and atrial filling A-wave (following a sinus P wave between two QRS deflections on a simultaneously-recorded electrocardiogram) were obtained, and E/A-ratio was calculated. To study left-ventricular (LV) contractile function, an M-mode echocardiogram was obtained at the level of the papillary muscles in the parasternal long axis view. LV diameters in both cardiac end-systole (defined as the smallest diameter) and end-diastole (defined as the largest diameter) were measured and LV volume calculated by the Teichholz method. LV ejection fraction was calculated as (systolic-volume minus diastolic-volume)/systolic-
volume×100%. The average of three to six cardiac cycles was used for each measurement, with the operator blinded to treatment assignment.

**Electrophysiological Study**

Closed-chest electrophysiological study was performed at two time points, pre-RFA (to confirm remodeling) and at terminal study, for CTL, ATR, and CHF groups. During electrophysiological studies, tachypacemakers were deactivated in ATR and CHF dogs. In CAF dogs, AF was direct-current cardioverted for the electrophysiological study before RFA; however, direct-current cardioversion was not performed at the terminal study to avoid dislodgment and loss of atrial thrombus. Electrophysiological studies were performed under sinus rhythm in all groups of dogs (and were therefore not performed at the terminal study in CAF dogs). Effective refractory period (longest S1S2-interval failing to capture) in the RA appendage was measured (2×threshold-current, 2-ms pulses, mean of 3 determinations) at basic cycle lengths of 150, 200, 250, 300, and 360 ms with 10 basic stimuli (S1s), followed by premature extrastimuli (S2s) with 5-ms decrements. To measure AF duration as an index of AF-promotion by remodeling, AF was induced with 1-10 s burst pacing (10-Hz, 4×threshold-current). Mean AF duration in each dog was based on 10 AF inductions for AF duration <20 min and 5 inductions for AF duration of 20-30 min. Prolonged AF (>30 min) was terminated by direct-current cardioversion. A 20-min rest period was allowed before continuing measurements. If prolonged AF was induced twice, no further AF induction was performed.

**Radiofrequency Ablation (RFA) Procedure**

Before RFA, ventricular pacemakers were deactivated in CHF-dogs to avoid hypotension and shock. RFA was performed during sinus rhythm in CTL, ATR-SR, and CHF-SR subgroups,
during atrial-tachypacing in ATR-AT and CHF-AT subgroups, and during AF in the CAF group. Dogs were anesthetized with morphine (morphine, 2 mg/kg, s.c.) and α-chloralose (120 mg/kg, i.v. load, 29.25 mg/kg/h), and ventilated mechanically. A non-irrigated 7F quadripolar radiofrequency catheter (Biosense Webster) with a 4-mm distal electrode tip was introduced via the right femoral vein and positioned under fluoroscopic and intracardiac echocardiographic guidance. Access to the LA was obtained by transeptal puncture. RFA lesions were produced by applying radiofrequency energy (50 W, target temperature 70°C) for 60 seconds. Maximum impedance-value, temperature, and power-output were displayed on the generator (EPT-1000XP, EP Technologies Inc) and recorded. Mean values were 128.1±1.7 Ω, 68.5±0.2°C and 13.4±0.6 W respectively. Four regions were targeted in each atrium and total of 8 lesions were created in each dog: LA appendage, LA inferior free wall, LA superior posterior wall, LA inferior posterior wall, RA superior septal wall, RA inferior septal wall, RA superior lateral wall, and RA inferior lateral wall. Although the sheaths were initially flushed with heparinized saline (1,000 IU/L), no other anticoagulation was used during the protocol.

**Blood Samples**

Citrated blood samples from a femoral vein were obtained at the terminal study, and subjected to analysis of thrombosis-related factors. The blood samples were centrifuged (3,000 rpm, 20 min, 4°C), the supernatant plasma removed and stored at -80°C until assay. The concentrations of a coagulation activation marker, thrombin-antithrombin complexes, and an inflammation marker, C-reactive protein, were analyzed with commercial ELISA kits.
**Histological and Histochemical Changes**

Animals were sacrificed at 7 days post-RFA after the terminal study. After sacrifice, the hearts were removed, fixed in 10% formalin and subjected to histological processing. Photographs of epicardial and endocardial surfaces were taken and gross surface maximal length and width of the ablation lesions were measured. Fixed tissues were dehydrated and embedded in paraffin. Specimens were serially sectioned perpendicular to the endocardial surface at a thickness of 6 µm with a motorized microtome (Olympus 4060E). Sections were stained with Masson’s trichrome and thrombi defined/quantified as described previously. A calibrated light microscope (Image-Pro Plus software, Bethesda, MD) was used for morphometric measurements of thrombus volume and lesion area/volume. Personnel analyzing lesion morphometry were blinded to experimental group. Mean thrombus volume and total thrombus volume were calculated in each dog for both atria, as well as LA and RA individually. Thrombus volume normalized to ablation lesion area and thrombus volume normalized to ablation lesion volume were also calculated for each lesion and then averaged in each dog for both atria, LA and RA.

**Data Analysis**

Continuous variables were assessed for normality using the Shapiro-Wilk test. Normally-distributed variables are expressed as mean±SEM. Non-normally distributed variables are expressed as medians and the Q1-Q3 (first and third interquartile values delimiting the bottom 25% and top 25% of the distribution) along with minimum/maximum values to describe sample-value variability. Multiple-group comparisons were obtained with 1-way ANOVA and Bonferroni-adjusted t-tests (for normally-distributed data) or Kruskal-Wallis test with Dunn's Multiple Comparison test (for non-normally distributed data) for non-repeated measures (main-effect factor dog-group). Repeated-measures analyses were performed for normally-distributed
data with 2-way ANOVA and Bonferroni-adjusted t-tests. Two-tailed $P<0.05$ indicated statistical significance. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

References


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LVDd=left ventricular end diastolic dimension; LVDs=left ventricular end systolic dimension; LVEF=left ventricular ejection fraction; LAAs=left atrial systolic area; LAAd=left atrial diastolic area; LAFAC=left atrial fractional area change; LADs=left atrial systolic dimension; LADd=left atrial diastolic dimension; LAFS=left atrial fractional shortening. Mean±SEM. *P<0.01, **P<0.001, and ***P<0.001 vs. CTL. †P<0.01 and ††P<0.01 vs. before RFA. ‡ A wave was absent because of AF. CTL=control; ATR=atrial tachycardia remodeling; CHF=congestive heart failure; SR=sinus rhythm; AT=atrial tachycardia; CAF=chronic atrial fibrillation. n=16, 8, 8, 7, 7, and 8 for CTL, ATR-SR, ATR-AT, CHF-SR, CHF-AT, and CAF, respectively.
### Supplemental Table 2.

#### All Left and Right Atrial Lesions

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<th>CHF-SR (n=7)</th>
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<td><strong>Ablation lesion area (mm²)</strong></td>
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<td>53.0±4.6</td>
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<td>56.7±7.5</td>
<td>44.3±3.7</td>
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<td><strong>Ablation lesion depth (mm)</strong></td>
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<td>4.7±0.3</td>
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<td><strong>Ablation lesion volume (mm³)</strong></td>
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<td>240.1±47.2</td>
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<td><strong>Number of thrombi per dog</strong></td>
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<td><strong>Presence of thrombus (%)</strong></td>
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<td>67.1±6.2</td>
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<td><strong>Mean thrombus volume (mm³)</strong></td>
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#### Left Atrial Lesions

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<td><strong>Ablation lesion area (mm²)</strong></td>
<td>43.7±7.5</td>
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<td><strong>Ablation lesion depth (mm)</strong></td>
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<td><strong>Presence of thrombus (%)</strong></td>
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#### Left Atrial Appendage Lesions

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<td>84.4±6.6</td>
<td>81.3±6.3</td>
<td>88.1±7.9</td>
<td>88.1±5.7</td>
<td>100.0±0.0</td>
</tr>
<tr>
<td>Mean thrombus volume (mm³)</td>
<td>30.1±5.4</td>
<td>26.5±7.2</td>
<td>18.8±4.9</td>
<td>15.9±6.2</td>
<td>19.9±5.7</td>
<td>32.8±8.3</td>
</tr>
</tbody>
</table>

CTL=control; ATR=atrial tachycardia remodeling; CHF=congestive heart failure; SR=sinus rhythm; AT=atrial tachycardia; CAF=chronic atrial fibrillation.
Data are mean±SEM.
Supplemental Figure legends

Supplemental Figure 1. Electrophysiologic characteristics of each subgroup of dogs pre-RFA and at terminal study. A and B, atrial effective refractory period (ERP, A) and AF duration (B) pre-RFA. C and D, atrial ERP (C) and AF duration (D) at terminal study. ERP values were normally-distributed and shown as mean±SEM. AF duration was not normally distributed and values are shown as median, Q1-Q3 box-plot, minimum and maximum value-whiskers. CTL=control; ATR=atrial tachycardia remodeling; CHF=congestive heart failure; SR=sinus rhythm; AT=atrial tachycardia; CAF=chronic atrial fibrillation. *P<0.005, **P<0.01, and ***P<0.001 for group differences vs. CTL. n=16, 8, 8, 7, 7, and 8 for CTL, ATR-SR, ATR-AT, CHF-SR, CHF-AT, and CAF, respectively.

Supplemental Figure 2. Plasma coagulation activation and inflammation markers in each subgroup of dogs. A, plasma thrombin-antithrombin complexes (TAT) concentration. B, plasma C-reactive protein (CRP) concentration. Values are shown as median, Q1-Q3 box-plot, minimum and maximum value-whiskers. n=16, 8, 8, 7, 7, and 8 for CTL, ATR-SR, ATR-AT, CHF-SR, CHF-AT, and CAF, respectively.

Supplemental Figure 3. Representative examples of histopathology and the size of thrombus in each subgroup. Representative images of Masson’s trichrome-stain in dogs from CTL (A), ATR-SR (B), ATR-AT (C), CHF-SR (D), CHF-AT (E), and CAF (F) subgroups. Arrows indicate thrombus formation at ablation sites. Thrombus sizes were 10 mm³, 25 mm³, 18 mm³, 11 mm³, 6 mm³, and 23 mm³, respectively. G, mean thrombus volumes in each subgroup of dogs. Values are shown as median, Q1-Q3 box-plot, minimum and maximum value-whiskers. n=16, 8, 8, 7, 7, and 8 for CTL, ATR-SR, ATR-AT, CHF-SR, CHF-AT, and CAF, respectively.
Supplemental Figure 1

A. Pre-RFA

B. Pre-RFA

C. Terminal study

D. Terminal study

Legend:
- CHF-SR
- CHF-AT
- CTL
- ATR-SR
- ATR-AT
- CAF

Graphs show ERP (ms) and AF duration (s) as functions of BCL (ms) for different conditions.
Supplemental Figure 2
Supplemental Figure 3