Activated factor IX, factor XI and tissue factor identify patients with permanent atrial fibrillation treated with warfarin who are at risk of ischemic stroke

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Abstract

Introduction: Previously, we have demonstrated that significant proportions of patients with various cardiovascular diseases have active tissue factor and active factor XIa in their plasma. In the current study, we evaluated active tissue factor and active factors (F)XI and FIX in plasma from patients with atrial fibrillation.

Material and methods: In 110 consecutive patients with permanent atrial fibrillation receiving warfarin, we determined active tissue factor, together with plasma FIXa and FXIa, using clotting assays by measuring the response to inhibitory monoclonal antibodies.

Results: Sixteen (14.5%) patients had detectable active tissue factor and active FXIa, including 11 subjects with both factors, while FIXa was observed in 28 (25.7%) patients. The three positive groups did not differ from the patients without these factors with regard to demographic and clinical characteristics. Von Willebrand factor was higher in the active tissue factor-positive group (p < 0.0001) and FXIa-positive group (p = 0.0037). Individuals positive for active tissue factor and FXIa had higher plasma interleukin-6 levels (p = 0.0014 and 0.0322, respectively). The presence of active tissue factor, FXIa and FIXa in anticoagulated patients with permanent atrial fibrillation correlated with elevated von Willebrand factor and interleukin-6. During a 3-year follow-up, ischemic stroke (n = 12, 10.9%) occurred more commonly among atrial fibrillation patients who had circulating TF (p = 0.002) or FXIa (p = 0.013).

Conclusions: These data suggest that circulating active coagulation factors, in particular TF and FXIa, can be detected despite oral anticoagulation in a significant proportion of patients with atrial fibrillation, and could represent novel markers of persistent prothrombotic alterations predisposing to ischemic stroke.

Key words: anticoagulation, arrhythmia, blood coagulation, stroke.

Introduction

Atrial fibrillation (AF) is associated with a prothrombotic state and an increased risk of thromboembolic complications [1, 2]. Mechanisms underlying hypercoagulability in AF are complex and involve hemodynamic changes, endothelial damage reflected by increased von Willebrand factor (VWF) antigen, enhanced platelet activation reflected by elevated circulating levels of β-thromboglobulin [3] and P-selectin [4], increased
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**Material and methods**

**Study population**

We studied 110 consecutive patients with permanent AF receiving oral anticoagulation with warfarin for more than 1 year after having screened 170 individuals with the time in the therapeutic range above 70% in the last year. We excluded patients with acute coronary syndrome within the preceding 6 months, heart failure (NYHA III/IV), hepatic dysfunction, chronic renal disease above level 3 (glomerular filtration rate less than 60 ml/min/1.73 m²), history of venous thromboembolism or stroke, acute illness, cancer, and those who underwent surgery within the preceding month – all the conditions associated with increased thrombin generation. Coronary artery disease (CAD) was defined as a history of myocardial infarction, coronary revascularization, or hospitalization for unstable angina. Diabetes was diagnosed according to the World Health Organization criteria. Hypercholesterolemia was defined as total cholesterol > 5 mmol/l, or low-density lipoprotein (LDL) cholesterol > 3.0 mmol/l or treatment with cholesterol-lowering medication. Arterial hypertension was defined according to a standard definition, i.e. systolic or diastolic pressure ≥ 140 mm Hg or ≥ 90 mm Hg, respectively, on at least two different measurements, previous history of hypertension or current antihypertensive treatment. Heart failure was diagnosed in subjects with decreased left ventricular ejection fraction (LVEF) < 50% on echocardiography. Thoracic echocardiography was performed in each patient using conventional techniques to measure the left atrial diameter and LVEF.

A 3-year follow-up started at the time of enrollment and was carried out on a 6-month basis by means of a visit to the center or telephone contact. None of the patients declared any interruption of warfarin use for more than 7 days. We recorded the objectively documented ischemic stroke.

The study was approved by the Ethical Committee, and all patients provided written informed consent.

**Laboratory methods**

Blood was drawn from an antecubital vein with minimal stasis after an overnight fast between 7 and 9 a.m. Serum and citrate plasma samples (9 : 1 of 3.2% sodium citrate) were centrifuged at 2,540 g for 15 min at 24°C within 20 min of collection, immediately frozen, and stored in aliquots at –80°C until further use. Lipid profiles, blood cell counts, glucose, creatinine, and International Normalized Ratio (INR) were assayed by routine laboratory techniques. High-sensitivity CRP was measured by latex nephelometry (Dade Behring,
Marburg, Germany). Commercially available immunoenzymatic assays were used to determine plasma IL-6 (R&D Systems, Abingdon, UK) and VWF antigen (Diagnostica Stago, Asnieres, France). All intra-assay and inter-assay coefficients of variation were below 7%.

**Plasma clotting assays**

Plasma was thawed at 37°C in the presence of 0.1 mg/ml corn trypsin inhibitor (CTI; prepared as previously described) [18]. CaCl₂ to a final 15 mM concentration was added and the plasma incubated for 1 min; clotting was initiated by the addition of 2 μM phospholipid vesicles (PCPS) composed of 25% dioleoyl-sn-glycero-3-phospho-L-serine and 75% of 1,2-dioleoyl-sn-glycero-3-phosphocholine (both from Avanti Polar Lipids, Inc; Alabaster, AL, USA) and prepared as described previously [19]. In parallel, inhibitory monoclonal anti-FXI (αFXI-2), anti-FIX (αFIX-91) or anti-TF (αTF-5) antibodies (both produced in house) at a final 0.1 mg/ml concentration were individually added to the same plasma prior to CaCl₂ addition. αFXI-2 is specific for FXI/XIa and inhibits FIX activation by FXla [20]. αTF-5 binds specifically to TF and interferes with TF/FVIIa complex formation [21]. Clotting times were determined using the ST8 instrument (Diagnostica Stago, Parsippany, NJ, USA). FXla, FIXa and TF activity in plasma was calculated from calibration curves developed with human FIXa or FXa (gifts from Dr. R. Jenny from Haematologic Technologies, Inc., Essex Junction, VT, USA) or re-lipidated [18] TF₁₋₂₄₃ (a gift from Dr. R. Lundblad from Baxter Healthcare Corp., Duarte, CA, USA) in pooled 10-donor normal plasma. The detectability limit for TF was 0.4 pM, for FXla 10 pM, and for FIXa 100 pM. In 12 healthy subjects all the 3 parameters were undetectable.

**Statistical analysis**

Due to skewness of most variables, continuous variables are presented as median and quartiles. Categorical variables are presented as numbers and percentages. To examine differences between two independent groups, the Mann-Whitney test, Welch’s t-test or the pooled t-test was used depending on normality of distributions and equality of variances of groups. The Fisher exact test was used to compare categorical variables. Odds ratios with 95% confidence intervals were computed using a logistic regression model with the presence of TF or FXla or FIXa (composite variable) as the dependent variable [22, 23]. The same method was performed to calculate stroke predictors. Two-sided p-values < 0.05 were considered statistically significant. Statistical analysis was performed with the use of JMP 9.0.0.

**Results**

Patient characteristics are presented in Table I. The study population was divided into groups with or without the specific coagulation factors, i.e.: TF(+) and TF(–), FXla(+) and FXla(–), FIXa(+) and FIXa(–). Age, prevalence of coronary artery disease, diabetes mellitus, heart failure, CHA₂DS₂-VASc score along with cholesterol, glucose, creatinine, INR, and CRP were similar in the TF(+) and TF(–) group, the FXla(+) and FXla(–) group, and the FIXa(+) and FIXa(–) group.

Tissue factor – 16 (14.5%) patients had detectable TF and the median value for only those who have > 0.4 pM TF was 1.17 pM (IQR: 0.43–2.19). Arterial hypertension was less common in the TF(+) group as compared to the TF(–) group. The proportion of current smokers was lower in the TF(+) group. In the CHADS₂ score, fewer patients had 1 point in the TF(+) group. Higher levels of VWF (+31.0%) and IL-6 (+40.1%) were observed in the TF(+) group compared with the TF(–) group. CRP was similar in both groups (Table I).

Factor XIa – 16 (14.5%) participants had measurable FXla (median 77.98 pM, IQR: 30.79–170.08). Both TF and FXla were observed in 11 (10%) subjects. Current smokers was less frequently observed in the FXla(+) group. Fewer individuals in the FXla(+) group had one point in the CHADS₂ score. VWF (+18.7%) and IL-6 (+21.3%) were higher in the FXla(+) group compared with the FXla(–) group. CRP was similar in both groups (Table I).

Factor IXa (FIXa) was observed in 28 (25.5%) patients with a median value of 592.4 pM (IQR: 209.0–1032.5). None of the patients had FIXa in combination with both TF and FXla. Males were more prevalent in the FIXa(+) group. Body mass index (BMI) was higher in the FIXa(+) group. A similar proportion of patients had 2 or more points in the CHADS₂ score. IL-6, VWF and CRP were similar in both groups (Table I).

The univariate logistic regression showed that a higher risk of the presence of one of the tested factors (TF, FXla or FIXa) was observed in patients with coronary artery disease (OR = 6.27; 95% CI: 1.85–28.9). Higher VWF (OR = 1.15; 95% CI: 1.05–1.27) and IL-6 (OR = 1.29; 95% CI: 1.05–1.61) were also associated with a higher risk of presence of one of the tested coagulation factors (Figure 1).

**Stroke** – During a 3-year follow-up we recorded 12 (10.9%) patients with ischemic stroke. No major bleeding events were observed; 10 patients had clinically relevant bleeding mostly from the gastrointestinal tract. The prevalence of stroke was higher in patients with diabetes, heart failure and in patients with two or more points in the CHADS₂ score (Table II). Higher levels of VWF (+18.4%), D-dimer (+51.1%), and fibrinogen (+27.4%) were
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| Table I. Characteristics of patients with chronic atrial fibrillation |
|-----------------|-----|-----|-----|-----|-----|-----|-----|
| Variable        | N = 110 | TF(+) n = 16 | TF(-) n = 94 | FXIa(+) n = 16 | FXIa(-) n = 94 | FIXa(+) n = 28 | FIXa(-) n = 81 |
| Age [years]     | 63.0 (55.0; 70.0) | 60.0 (52.0; 67.8) | 63.0 (56.0; 70.3) | 57.0 (50.3; 69.8) | 63.5 (56.8; 70.0) | 61.5 (53.3; 66.8) | 64.0 (55.0; 71.5) |
| Male gender, n (%) | 57 (51.8) | 8 (50.0) | 49 (52.1) | 7 (43.8) | 50 (53.2) | 20 (71.4)* | 37 (45.7) |
| BMI [kg/m²]     | 29.1 (26.6; 31.4) | 30.7 (26.9; 32.2) | 29.0 (26.3; 31.2) | 27.1 (25.1; 32.0) | 29.2 (26.8; 31.2) | 31.8 (26.4; 35.5)* | 28.4 (26.6; 30.6) |
| Hypertension, n (%) | 65 (59.1) | 4 (25.0)* | 61 (64.9) | 6 (37.5) | 59 (62.8) | 19 (67.9) | 46 (56.8) |
| CAD, n (%)      | 15 (13.6) | 3 (18.8) | 12 (12.8) | 4 (25.0) | 11 (11.7) | 7 (25.0) | 8 (9.9) |
| Diabetes, n (%) | 6 (5.5) | 2 (12.5) | 4 (4.3) | 2 (12.5) | 4 (4.3) | 1 (3.6) | 5 (6.2) |
| Heart failure, n (%) | 20 (18.2) | 1 (6.3) | 19 (20.2) | 1 (6.3) | 19 (20.2) | 7 (25.0) | 13 (16.0) |
| Smoking, n (%)  | 44 (40.0) | 2 (12.5)* | 42 (44.7) | 2 (12.5)* | 42 (44.7) | 10 (35.7) | 34 (42.0) |
| CHADS2 ≥ 2 points | 27 (24.5) | 3 (18.8) | 25 (25.5) | 4 (25.0) | 23 (24.5) | 7 (25.0) | 20 (24.7) |
| CHADS2 ≥ 2 points, n (%) | 47 (42.7) | 2 (12.5)* | 45 (47.9) | 2 (12.5)* | 45 (47.9) | 15 (53.6) | 32 (39.5) |
| CHA2DS2-VASc ≥ 1 point, n (%) | 64 (58.2) | 5 (31.3)* | 59 (62.8) | 6 (37.5) | 58 (61.7) | 16 (57.1) | 48 (59.3) |
| Aspirin, n (%)  | 35 (31.8) | 9 (56.2)* | 26 (27.7) | 12 (75.0)* | 23 (24.5) | 12 (42.9) | 22 (27.2) |
| Statin, n (%)   | 53 (48.2) | 1 (6.3)* | 52 (55.3) | 4 (25.0) | 49 (52.1) | 10 (35.7) | 43 (53.1) |
| Total cholesterol [mM] | 5.05 (4.17; 5.62) | 5.01 (4.62; 5.89) | 5.05 (4.01; 5.61) | 4.83 (4.05; 5.49) | 5.08 (4.18; 5.65) | 5.25 (3.91; 5.87) | 5.03 (4.19; 5.59) |
| LDL cholesterol [mM] | 2.91 (2.36; 3.40) | 3.02 (2.51; 3.38) | 2.86 (2.36; 3.40) | 3.00 (2.34; 3.10) | 2.86 (2.38; 3.42) | 2.95 (2.22; 3.79) | 2.88 (2.41; 3.36) |
| HDL cholesterol [mM] | 1.46 (1.14; 1.70) | 1.61 (1.21; 1.74) | 1.44 (1.14; 1.69) | 1.56 (1.00; 1.69) | 1.45 (1.18; 1.71) | 1.46 (1.15; 1.72) | 1.45 (1.14; 1.70) |
| Triglycerides [mM] | 1.15 (0.74; 1.66) | 1.43 (0.64; 1.93) | 1.13 (0.75; 1.58) | 1.17 (0.62; 1.67) | 1.13 (0.75; 1.66) | 0.94 (0.67; 1.63) | 1.17 (0.78; 1.69) |
| Glucose [mM]    | 4.90 (4.50; 5.20) | 4.95 (4.53; 5.40) | 4.87 (4.50; 5.20) | 4.85 (4.43; 5.30) | 4.90 (4.43; 5.20) | 4.85 (4.43; 5.08) | 4.90 (4.51; 5.25) |
| Creatinine [µM] | 70.7 (60.8; 80.3) | 72.5 (58.0; 85.8) | 70.7 (61.0; 79.7) | 62.5 (50.8; 84.0) | 62.5 (61.9; 80.3) | 69.5 (60.0; 79.8) | 69.5 (60.0; 79.8) |
| INR              | 2.48 (2.25; 2.83) | 2.50 (2.20; 2.78) | 2.45 (2.25; 2.84) | 2.45 (2.39; 2.92) | 2.44 (2.20; 2.75) | 2.40 (2.08; 2.72) | 2.49 (2.30; 2.85) |
| VWF (%)          | 210.5 (169.0; 245.3) | 271.0 (251.0; 279.0)* | 196.5 (168.0; 231.3) | 253.0 (192.8; 278.8)* | 205.0 (168.0; 233.5) | 225.0 (168.5; 235.8) | 202.0 (169.0; 245.5) |
| CRP [mg/l]       | 2.96 (1.89; 4.44) | 2.35 (1.20; 4.13) | 3.01 (1.92; 4.70) | 2.23 (1.20; 4.13) | 3.01 (1.96; 4.51) | 3.12 (2.08; 5.29) | 2.98 (1.83; 4.42) |
| IL-6 [pg/ml]    | 5.55 (4.40; 7.03) | 7.65 (5.50; 9.88)* | 5.40 (4.30; 6.70) | 6.30 (5.30; 9.33)* | 5.40 (4.30; 6.83) | 5.40 (4.23; 7.08) | 5.60 (4.45; 6.75) |
| LVEF (%)         | 49.0 (41.2; 55.0) | 52.1 (41.5; 59.7) | 47.9 (40.9; 55.0) | 43.3 (43.3; 59.7) | 47.9 (40.4; 55.0) | 51.0 (42.4; 57.9) | 48.0 (40.8; 53.4) |
| LA [mm]          | 43.0 (40.0; 46.0) | 44.5 (42.3; 52.8)* | 43.0 (40.0; 45.0) | 42.5 (38.5; 44.8) | 43.0 (40.0; 46.0) | 43.5 (40.0; 47.0) | 43.0 (40.5; 45.0) |

Values are presented as median and quartiles or as percentages. *Values of p < 0.05 versus a subgroup without the factor tested.
observed in patients who experienced stroke. As expected, time in therapeutic range (TTR) (–27.4%) was lower in patients with stroke (Table II). Importantly, TF and FXIa were present more commonly among the patients with stroke despite warfarin use (OR = 8.80, 95% CI: 2.36–33.67, and OR = 5.65, 95% CI: 1.46–21.02, respectively) (Figure 2). FIXa tended to be more prevalent among patients with stroke (p = 0.07) (Table II). In the multivariate logistic regression model the independent predictors of stroke in AF patients while on warfarin were heart failure, treatment with angiotensin-converting enzyme inhibitor (ACEI) and the presence of active TF after adjustment for TTR.

**Discussion**

Our data indicate that TF, FXIa and FIXa are observed in a substantial proportion of anticoagulated AF patients. Permanent AF per se is a pathological state in which despite warfarin therapy enhanced blood coagulation reflected by the presence of active TF, FXIa and FIXa can be observed. The appearance of these factors is independent from INR values. Butenas et al. showed that CAD leads to increased levels of plasma active TF and FXIa and inflammatory cytokines are involved in this phenomenon [9]. In hypertensive individuals higher TF [10] and FIXa [24] have been observed. AF itself, being a hypercoagulable state, in association with diabetes enhances prothrombotic alterations, because it is known that diabetes is characterized by increased amounts of circulating TF, fibrinogen, and thrombin formation markers [25–27]. In search for determinants of the presence of active TF, FXI and FIXa in AF patients, we found that CAD is associated with the presence of one of the tested factors. One might speculate that this factor may contribute to the progression of this disease. We failed to observe any correlation between the three factors and diabetes. Unexpectedly, in the TF(+) group, hypertensive patients were underrepresented compared with the TF(–) group. Hypercholesterolemia has been suggested to induce TF expression, and statin treatment may reduce active TF in circulating blood [11, 28]. In our study, a substantial proportion of AF patients without TF were treated with statins. However, it should be stressed that statin intake characterizes patients at higher cardiovascular and thromboembolic risk. A novel finding is an association of VWF with the presence of active TF and FXIa in AF patients. Increased plasma VWF that results
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Table II. Characteristics of atrial fibrillation patients with and without stroke incidence during 3-year follow-up

| Variable | Stroke | P-value |
|----------|--------|---------|
|          | Absent (N = 98, 89.1%) | Present (N = 12, 10.9%) | |
| Diabetes, n (%) | 3 (3.1) | 3 (25.0) | 0.017 |
| Heart failure, n (%) | 14 (14.3) | 6 (50.0) | 0.008 |
| ACEI, n (%) | 25 (25.5) | 9 (75.0) | 0.001 |
| Fibrinogen [g/l] | 4.00 (2.38; 4.83) | 4.38 (3.91; 5.16) | 0.04 |
| VWF (%) | 201.5 (168.0; 236.8) | 238.5 (210.8; 278.0) | 0.006 |
| D-dimer [ng/ml] | 215.0 (154.8; 277.5) | 317.0 (287.5; 404.0) | 0.0001 |
| TF present, n (%) | 10 (10.2) | 6 (50.0) | 0.002 |
| FIXa present, n (%) | 11 (11.2) | 5 (41.7) | 0.015 |
| FIIa present, n (%) | 22 (22.7) | 6 (50.0) | 0.073 |
| FIXa, FIXa or TF present, n (%) | 37 (37.8) | 12 (100) | < 0.0001 |
| Time in therapeutic range (%) | 75.0 (65.0; 85.0) | 55.0 (45.0; 60.0) | < 0.0001 |
| CHADS2, 1 point, n (%) | 47 (48.0) | 0 (0.0) | 0.0011 |
| CHADS2, ≥ 2 points, n (%) | 18 (18.4) | 9 (75.0) | 0.0001 |

Abbreviations see Table I. ACEI – angiotensin-converting enzyme inhibitor.

Figure 2. Univariate logistic regression analyses for ischemic stroke in anticoagulated patients with permanent atrial fibrillation during follow-up. Stroke is associated with the use of ACEI, higher CHADS2 score, vWF, D-dimer, fibrinogen, coexisting diabetes type 2 or heart failure, and detection of TF or FIXa at enrolment. Only higher TTR is associated with low stroke risk.

The graph with logarithmic scale shows point estimates of odd ratios with 95% confidence intervals. ACEI – angiotensin-converting enzyme inhibitor, vWF – von Willebrand factor, LA – left atrium size, TTR – time in therapeutic range, F – factor.

*p < 0.05.
from endothelial damage has been demonstrated to predict cardiovascular events including stroke in AF [29]. It originates from damaged endothelial cells but in AF mostly from atrial endothelial cells [30]. The current findings provide additional evidence that there is a correlation between endothelial dysfunction and prothrombotic state reflected by the activity of TF and FXI in AF and this link is present despite oral anticoagulation.

Our data showed that IL-6 was higher in anticoagulated AF patients with detectable TF or FXIa. It is well known that inflammation is associated with AF, and ongoing inflammation can predispose to AF. Moreover, structural heart diseases can promote inflammation, and inflammatory markers are increased in this arrhythmia [31–33]. Since IL-6 failed to be shown as an independent predictor of the presence of TF or FXIa in our patients, it might be speculated that in permanent AF during warfarin administration inflammation has a minor effect on the generation and persistence of active TF and FXIa.

To our knowledge, our study is the first to show the presence of circulating plasma FXIa in chronic AF and characterizes factors associated with the presence of circulating TF or FXIa. The current study suggests that regulation of FXIa formation and persistence in circulating blood differ from the mechanisms and associations observed for active TF and FXIa in AF patients. Due to the paucity of data on FXIa in the plasma of cardiovascular patients, its role in blood coagulation merits further investigations.

We observed that stroke occurs more commonly among anticoagulated AF patients who had circulating TF and FXIa at enrollment. This novel finding suggests that the two coagulation factors present on warfarin, together with low quality of anticoagulation, heart failure and diabetes, predispose to ischemic complications of AF during follow-up. Larger studies are needed to validate this observation.

Several study limitations should be acknowledged. The small number of the patients studied especially in subgroups hampers the data interpretation. A larger cohort is needed to confirm our observations. There was no control ‘disease’ group with sinus rhythm; therefore we cannot exclude a potential impact of coexisting cardiovascular diseases or medications on the presence of circulating TF, FXIa or FXIa. Since transesophageal echocardiography was not performed, we cannot exclude the presence of a thrombus within the left atrium appendage, which might increase hypercoagulability including circulating TF. Long-term follow-up with clinical outcomes is needed to evaluate the potential predictive value of active TF, FXIa, or FXIa in thromboembolic risk associated with permanent AF. It remains to be established whether new oral thrombin and FXIa inhibitors, which are now widely used in AF, can more effectively suppress formation of active TF, FXIa, and FXIa [34, 35]. A role of these factors, particularly TF, cannot be ruled out also in other cardiovascular diseases, e.g. abdominal aneurysm [36].

In conclusion, the present study showed the presence of TF, FXIa and FXIa in anticoagulated patients with permanent AF. VWF and IL-6 have been identified as laboratory determinants of the presence of those active coagulation factors in this AF patient population despite anticoagulation. It is tempting to speculate that TF, FXIa and FXIa might be considered as novel markers of thromboembolic risk in AF, which can be detected even during warfarin therapy.

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Conflict of interest

The authors declare no conflict of interest.

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