Interleukin-6, endothelial activation and thrombogenesis in chronic atrial fibrillation

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Background A prothrombotic or hypercoagulable state has been described in AF, which could increase the risk of thromboembolism. As inflammation has been related to thrombogenesis and endothelial activation, we hypothesised that the prothrombotic state in AF (as assessed by an index of thrombogenesis, prothrombin fragment 1+2 [F1+2]) and endothelial activation (soluble E-selectin (sEsel)) could be related to an index of inflammation (interleukin-6 (IL-6)).

Patients and methods We studied 191 consecutive patients (98 male; mean age 72.3±9.2 years) with chronic non-rheumatic AF who were not on anticoagulant therapy. Plasma IL-6, sEsel and F1+2 were measured by ELISA. Research indices were compared to 74 controls in sinus rhythm matched for age and sex. In 43 patients with AF, the effects of introducing anticoagulation (INR 2.0–3.0) were also studied.

Results Patients with AF had elevated levels of F1+2 (p<0.001) and IL-6 (p=0.045), but not sEsel. There was no significant correlation between F1+2 and IL-6. In multivariate analysis, only F1+2 levels were independently associated with the presence of AF (p=0.001). After oral anticoagulation, plasma levels of F1+2 and sEsel were significantly decreased (both p<0.01).

Conclusion High levels of IL-6 in AF suggest an inflammatory state, which appears to be more related to clinical variables of the patients, rather than to the presence of AF per se. There was no association of inflammation with endothelial activation (sEsel) or the presence of abnormal thrombogenesis (high F1+2 levels) in AF. Moreover, no changes in IL-6 levels were found despite the reduction of the other markers by oral anticoagulant therapy.

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Introduction

Abnormalities of haemostasis, fibrinolysis, endothelium and platelets have been described in atrial fibrillation (AF), which may increase the risk of stroke and thromboembolism in this common cardiac arrhythmia.1−4 This prothrombotic state has been associated with left atrial thrombus and spontaneous echo contrast.5 The hypercoagulable state observed in AF may be additive to the presence of clinical and echocardiographical risk factors for thromboembolism.6−8
There is an apparent link between thrombogenesis and inflammation.\textsuperscript{9–11} For example, the Speedwell Study reported a correlation between fibrin D-dimer (an index of fibrin turnover) and CRP levels, which may have associations with risk of ischaemic heart disease.\textsuperscript{12} Interestingly, elevated CRP levels have been reported in AF patients, reflecting an inflammatory state, which could promote the persistence of AF.\textsuperscript{13} However, to the best of our knowledge, there are no studies relating the hypercoagulable state described in AF to inflammatory markers.

An established index of inflammation is interleukin-6 (IL-6), which is a circulating cytokine produced by monocytes, macrophages, T lymphocytes and endothelial cells. IL-6 can induce a prothrombotic state by increasing expression of fibrinogen, tissue factor, factor VIII and von Willebrand factor, as well as by activating endothelial cells and increasing platelet production.\textsuperscript{10} Indeed, inflammation leads to the adhesion, recruitment and trans-endothelial migration of leucocytes into the intima, which is mediated by adhesion molecules on the endothelial cell membrane; leading to the initial capture and rolling of leucocytes along the endothelium.\textsuperscript{14} In particular, E-selectin is a specific endothelial cell product which mediates contact between endothelial cells and neutrophils and monocytes.\textsuperscript{15} There are two important features regarding E-selectin expression: it is generally absent in normal tissues, and it has not been seen on cells other than the endothelium.\textsuperscript{16} E-selectin may be induced in vitro by inflammatory cytokines and shed (hence, measurable soluble E-selectin levels [sEsel]) which probably reflects immunological activation of the endothelium.\textsuperscript{17} Raised levels of sEsel have been reported in hypertension,\textsuperscript{18} diabetes\textsuperscript{19} and in atherosclerotic vascular disease, such as stroke\textsuperscript{20} and ischaemic heart disease.\textsuperscript{21}

As inflammation has been related to thrombogenesis and endothelial activation, we hypothesised that the prothrombotic state in AF (as assessed by indices of thrombogenesis, prothrombin fragment 1+2 [F1+2]) and endothelial activation (sEsel) is related to inflammation (marked by interleukin-6, IL-6). To test this hypothesis, we performed a cross-sectional study of 191 patients with AF who were compared to (age and sex) matched controls in sinus rhythm with broadly similar comorbidity. Additionally, we also studied the influence of oral anticoagulation on these markers.

**Patients and methods**

We studied 191 consecutive patients (98 male; 72.3±9.2 years) with non-rheumatic AF lasting more than 4 weeks (as documented by electrocardiograms), who were referred by general practitioners or cardiologists to our anti-coagulation clinic for the initiation of anticoagulation treatment with acenocoumarol. None of the patients had previously been on anticoagulant therapy, although 83 were taking aspirin. Risk factors for thromboembolism were recorded — both clinical (age, sex, hypertension, diabetes, heart failure and previous embolism) and echocardiographic variables (left atrial diameter, spontaneous echocontrast and left ventricle ejection fraction).

We excluded patients with recent (<3 months) venous or systemic thromboembolism, myocardial infarction, stroke or acute coronary syndrome, infection or inflammatory disease, and/or surgery; malignancy and renal/liver impairment. Finally, we also excluded patients with valvular heart disease and those being treatment with hormone replacement therapy or oral anticoagulation. Patients with AF were compared to 74 matched controls in stable sinus rhythm (with broadly similar comorbidity, but without any history of AF) who were recruited from hospital staff, patient relatives and those attending hospital for minor procedures (hernia, cataract, etc).

In 43 patients with AF, blood samples were obtained at baseline and after 3 months of steady therapeutic anticoagulation (INR 2–3). Oral anticoagulant therapy was monitored using an automated assay (ACL Futura, Instrumentation Laboratory Co.) with a bovine calcium thromboplastin (Pro-IL-Complex, ISI 1.02). We recorded the acenocoumarol dose and the INR at the moment of blood sampling.

**Blood samples and laboratory assays**

Venopuncture was performed in the morning on patients, who had been fasting for >12 h and had rested for at least 20 min. Blood samples were drawn atraumatically and without stasis into syringes preloaded with trisodium citrate (0.011 M). Platelet poor plasma fractions were obtained by centrifugation at 4 °C for 20 min at 2200 g (within 5 min after blood collection). Aliquots were stored at −30 °C to allow batch analysis. Soluble E-selectin and IL-6 were measured by ELISA with R&D Systems reagents (Abingdon, UK), with a minimum sensitivity of 1.6 ng/ml for sEsel and 2.5 pg/ml for IL-6. F1+2 levels were assayed by ELISA using a commercial kit (Enzygnost F1+2.
micro, Dade Behring Marburg GmbH, Marburg, Germany) and following the manufacturer’s instructions. The intra-assay and inter-assay coefficients of variation were less than 5% and 10% respectively for all tests.

**Echocardiography**

Transthoracic M-mode, two-dimensional and Doppler echocardiography (Hewlett-Packard SONOS 2500, California, USA) was performed in all AF patients. All echocardiographic recordings were performed by the same investigator, and the coefficient of variation for our laboratory was <5%. Echocardiographic measures were performed in the long parasternal and apical four-chamber apical axis. Measurements were taken according to the guidelines laid down by the American Society of Echocardiography.22

**Power calculations**

We have previously demonstrated that 6 weeks of dose-adjusted warfarin reduced median levels of another index of thrombogenesis (fibrin D-dimer) by 50% among 51 patients with AF.23 However, in the current study, as we intended to measure additional indices, we more conservatively hypothesised a reduction in F1+2 levels of one-third of its pre-treatment (baseline) level. In order to achieve this difference at \( p < 0.05 \) and a 1-beta of 0.8, we needed good data from 25 subjects per intervention group. However, as stated, we recruited in excess of this figure to improve our chances of minimizing Types I and II errors.

**Statistical analysis**

Continuous variables were tested for normal distribution by Kolmogorov–Smirnov test. Data not normally distributed were log-transformed for analysis and are presented in the non-logarithmic format as median (25th–75th percentiles values). Data normally distributed are presented as mean and standard deviation. Comparisons between two groups was performed by Mann–Whitney \( U \) test, or Student’s \( t \)-test. Chi-square test was used to compare quantitative data between different groups. Paired comparisons were performed using the paired Wilcoxon test. Correlations were performed using Spearman’s rank correlation method. Multivariate analysis was performed by stepwise multiple regression analysis using the research indices as the dependent variable and clinical features as predictors. A two-tailed \( p \) value of <0.05 was considered as statistically significant. Analyses were performed using the SPSS statistical package for Windows version 10.0 (SPSS Inc, Illinois, USA).

**Results**

Clinical data of patients and controls are summarized in Table 1. AF patients showed elevated levels of F1+2 and IL-6, with no statistically significant differences in sEsel levels compared to controls. Women (\( p = 0.021 \)) and patients with previous stroke...
and/or peripheral embolism had higher levels of IL-6 \((p=0.001)\), whilst patients with heart failure had higher levels of F1+2 \((p=0.032)\) [Table 2]. There were no significant differences in F1+2, IL-6 and sEsel levels between patients who were taking aspirin or not, nor any association between research indices and echocardiographic variables (data not shown).

There were no significant correlations between F1+2, IL-6 and sEsel levels. In addition, there were no significant correlations between any of the research indices and clinical variables (i.e. age and duration of arrhythmia) (data not shown).

**Effects of anticoagulation**

After 3 months of steady state oral anticoagulation, there was a significant reduction in plasma levels of F1+2 \((p=0.001)\), and sEsel \((p=0.001)\), but no significant change in IL-6 levels (Table 3). There were no significant correlations between the research indices with daily dose of acenocoumarol or INR values (F1+2 and dose of acenocoumarol: \(r=0.109, p=0.496\); sEsel and dose of acenocoumarol: \(r=-0.078, p=0.622\); F1+2 and INR: \(r=-0.235, p=0.140\); sEsel and INR: \(r=-0.053, p=0.737\)).

**Multivariate analysis**

In stepwise multiple regression analysis of the AF group, IL-6 levels were associated with previous thromboembolism \((p=0.001)\) and sex \((p=0.018)\). There were no significant predictors for sEsel and F1+2 levels.

If data from the whole study group were entered into the stepwise multiple regression analysis, taking the presence/absence of AF as a predictor, F1+2 levels were independently associated with the presence of AF \((p=0.001)\). Again, IL-6 levels were associated with previous embolism and sex (all \(p>0.05\)). There were no significant predictors for sEsel levels.

**Discussion**

The presence of AF confers a hypercoagulable state, which appears to be unrelated to underlying heart disease.\(^1\) In our consecutive population of AF patients, we found that elevated levels of F1+2 were independently associated with the presence of AF on multivariate analysis. Nevertheless, it is unclear if this hypercoagulable state has an added value predicting stroke. In 553 AF patients participating in the Stroke Prevention in Atrial Fibrillation III Study (SPAF III), which were broadly similar to ours, Feinberg et al.\(^24\) found that high F1+2 levels were associated with clinical risk factors for stroke (age, female sex, systolic blood pressure and heart failure). For assessing the role of F1+2 as predictor of subsequent stroke or thromboembolism, the analyses from SPAF III also included 263 'low risk' patients who were taking aspirin and low (inefficacious) doses of warfarin, and had plasma F1+2 levels measured 3 months after study entry (as they had no sample at baseline). They found that plasma F1+2 levels were not associated with stroke and thromboembolism, after adjustment for age; nonetheless, there could some influence of their concomitant antithrombotic treatment as well as some selection bias, since many of the patients were at low thromboembolic risk.

Recently, an association between AF and CRP levels has been reported,\(^13\) which was independent of hypertension, structural heart disease or previous stroke or embolism. This study suggested that as CRP is a marker of an inflammatory state, the latter might promote the persistence of AF.\(^13\) This study included several types of atrial arrhythmias, and their definition of 'lone' atrial arrhythmia or AF included those without structural heart disease but could include those with hypertension or a previous stroke – features associated with inflammation.\(^25\) We did not measure CRP levels in our patients, but IL-6 has been demonstrated as a useful marker of inflammation,\(^10,26\) and a good predictor of cardiovascular disease.\(^27\) In the present study, our AF patients had higher levels of IL-6 on univariate analysis, but in the multivariate analysis, IL-6 was no longer independently related to the presence of AF, suggesting that it was probably related to the associated comorbidity of our study group.\(^28\) Nevertheless, there was an independent association between IL-6 and previous thromboembolism, and indeed, IL-6 could promote thrombosis by increasing the expression of TF, fibrinogen, factor VIII and von Willebrand factor.\(^10\) Despite a previous reported association between thrombogenesis and inflammation (i.e. Speedwell Study\(^12\)) we did not find any significant correlation between F1+2 and IL-6 levels in our AF patients.

In the present study, women had elevated levels of IL-6, and we are aware that oestrogen could repress IL-6 expression whilst the menopause can result in higher serum levels of IL-6.\(^29\) Nonetheless, plasma IL-6 levels have been shown to be independently related to clinical cardiovascular risk factors, including hormone therapy replacement.\(^30\) Elevation of CRP levels after hormone therapy replacement has also been reported.\(^31\) In the present study, women on hormone replacement therapy were excluded.
Table 2  Soluble E-selectin, interleukin 6, tissue factor, prothrombin fragment 1+2 in different sub groups of patients with atrial fibrillation

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>(a) Sex</th>
<th>(b) Age</th>
<th>(c) Diabetes mellitus</th>
<th>(d) Hypertension</th>
<th>(e) IHD(^d)</th>
<th>(f) Heart failure</th>
<th>Stroke/embolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>n≥65 years</td>
<td>&lt;65 years</td>
<td>n≥65 years</td>
<td>&lt;65 years</td>
<td>n≥65 years</td>
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<tr>
<td>sEsSel</td>
<td>98</td>
<td>93</td>
<td>158</td>
<td>33</td>
<td>0.196</td>
<td>0.930</td>
<td>0.980</td>
</tr>
<tr>
<td>(ng/ml)(^a)</td>
<td>(30.0–56.0)</td>
<td>(32.7–69.6)</td>
<td>(32.2–62.8)</td>
<td>(32.9–63.4)</td>
<td>(2.5–11.0)</td>
<td>(2.9–7.8)</td>
<td>(2.5–11.0)</td>
</tr>
<tr>
<td>IL-6</td>
<td>4.1 (2.5–7.5)</td>
<td>6.0 (2.5–12.5)</td>
<td>5.0 (2.5–11.0)</td>
<td>5.0 (2.9–7.8)</td>
<td>0.871</td>
<td>0.100</td>
<td>0.032</td>
</tr>
<tr>
<td>(pg/ml)(^b)</td>
<td>(1.00–1.85)</td>
<td>(1.04–2.06)</td>
<td>(1.05–2.03)</td>
<td>(0.95–1.75)</td>
<td></td>
<td></td>
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<tr>
<td>F1+2</td>
<td>1.36</td>
<td>1.29</td>
<td>1.36</td>
<td>1.21</td>
<td>0.871</td>
<td>0.100</td>
<td>0.032</td>
</tr>
<tr>
<td>(nmol/l)(^c)</td>
<td>(1.00–1.85)</td>
<td>(1.04–2.06)</td>
<td>(1.05–2.03)</td>
<td>(0.95–1.75)</td>
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<tr>
<td>sEsSel</td>
<td>44.0</td>
<td>44.0</td>
<td>45.5</td>
<td>44.0</td>
<td>0.550</td>
<td>0.032</td>
<td>0.732</td>
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<td>(ng/ml)(^a)</td>
<td>(32.4–62.8)</td>
<td>(30.8–63.9)</td>
<td>(31.2–62.0)</td>
<td>(31.2–64.0)</td>
<td>(1.02–1.81)</td>
<td>(1.02–2.01)</td>
<td>(1.07–2.58)</td>
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<tr>
<td>IL-6</td>
<td>4.6 (2.5–10.0)</td>
<td>5.0 (2.7–11.0)</td>
<td>5.0 (2.5–11.0)</td>
<td>4.8 (2.5–9.9)</td>
<td>1.37</td>
<td>1.33</td>
<td>1.29</td>
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<tr>
<td>(pg/ml)(^b)</td>
<td>(1.02–2.11)</td>
<td>(1.02–2.03)</td>
<td>(1.02–2.08)</td>
<td>(1.01–1.85)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1+2</td>
<td>1.43</td>
<td>1.31</td>
<td>1.44</td>
<td>1.6</td>
<td>0.381</td>
<td>0.662</td>
<td>1.25</td>
</tr>
<tr>
<td>(nmol/l)(^c)</td>
<td>(1.00–1.81)</td>
<td>(1.02–2.03)</td>
<td>(1.02–2.08)</td>
<td>(1.01–1.85)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\(^a\) sEsSel: soluble E-selectin.
\(^b\) IL-6: interleukin-6.
\(^c\) F1+2: prothrombin fragment 1+2.
\(^d\) IHD: ischaemic heart disease.
Endothelial activation in AF may lead to increased endothelium adhesiveness to leukocytes, the production of procoagulant and vasoactive molecules, cytokines and growth factors; eventually leading to endothelium dysfunction and finally damage.\textsuperscript{32,33} Endothelial dysfunction/damage has been demonstrated in AF patients, as one thrombotic mechanism of Virchow’s triad.\textsuperscript{1} Moreover, among 1321 AF patients participating in the SPAF III study, four clinical risk factors for stroke in AF (age, previous stroke, heart failure and diabetes) were independently associated with elevated von Willebrand factor plasma levels (a recognized marker of endothelial damage/dysfunction).\textsuperscript{4}

E-selectin is a specific endothelial product and raised levels are more considered to indicate endothelial activation rather than damage, and current data suggest no direct part of sEsel in either coagulopathy or thrombogenesis.\textsuperscript{34} To our knowledge, the present study is the first examining tests endothelial activation (sEsel levels) in AF, and we did not find any differences between patients and controls, possibly because of the ‘steady state’ of the disease at recruitment or the high frequency of co-morbidity and risk factors such as diabetes. Elevation levels of sEsel have been reported after ‘acute’ (endothelial?) activation states, such as stress stimuli (cold pressor test\textsuperscript{35} or exercise\textsuperscript{36}) reflecting an endothelial response; as well as in the acute phase of stroke\textsuperscript{20} or myocardial infarction/ unstable angina.\textsuperscript{37}

In the present study, dose-adjusted acenocoumarol significantly reduced plasma of F1+2, in keeping with a reduction in thrombogenesis and fibrin turnover.\textsuperscript{38} The improvement of the hypercoagulable state also seems to benefit the endothelium, as the plasma levels of sEsel were also significantly reduced. Similarly, a reduction of sEsel levels has been shown in several clinical settings, after reducing/removing the relevant risk factor.\textsuperscript{39,40} The marked (37%) fall in sEsel is surprising. The mechanism for this is unclear but may be direct (acenocoumarol influencing cell signalling) or indirect (vitamin K dependent proteins acting on the endothelium). As expected, anticoagulation did not influence IL-6 levels, nor was there any correlation between IL-6 and thrombogenesis (F1+2), suggesting that raised levels were more related to the comorbidity of the patients than to the presence of AF or the hypercoagulable state.

This study is limited by its cross-sectional design, which only allows us to explore associations, and no causality is implied, as only a prospective cohort study with large numbers of subjects with AF can confirm the natural history of the indices measured in the short, medium and long-term. Indeed, IL-6 is a key inflammatory cytokine, and is normally present in low concentrations (but increasing during infection, trauma, stress and age, etc); thus, an ability to determine the independent relationship between IL-6 and (say) AF would certainly require further longitudinal studies, as well as prolonged periods of ‘exposure’ to the particular disease state. In the present study, we did investigate a fairly large cohort of patients with AF, who had documented AF of >4 weeks. Finally, the endocardial surface could be considered as part of the systemic endothelium, and in this way could be a source of E-selectin, but the data differentiating between the two in relation to E-selectin are limited. However, the expression of E-selectin in endocardium has been studied in relation to human cardiac allograft rejection, but these studies demonstrated a little or no expression of E-selectin in endocardium specimens.\textsuperscript{41–43}

In conclusion, we have demonstrated slightly raised levels of IL-6 in AF which suggest the presence of an inflammatory state, although this appears to be more related to clinical variables of the patients, rather than to the presence of AF per se. Importantly, there was no association of inflammation with endothelial activation (sEsel) or

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Effects of introducing anticoagulation (INR 2.0–3.0) in patients with atrial fibrillation</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>43</td>
</tr>
<tr>
<td>Male (%)</td>
<td>20 (46.5%)</td>
</tr>
<tr>
<td>Age</td>
<td>74.1±8.3</td>
</tr>
<tr>
<td>sEsel (ng/ml)\textsuperscript{a}</td>
<td>50.0 (38.0–70.0)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)\textsuperscript{b}</td>
<td>7.0 (3.1–11.0)</td>
</tr>
<tr>
<td>F1+2 (nmo/l)\textsuperscript{c}</td>
<td>1.24 (1.00–2.03)</td>
</tr>
<tr>
<td>After 3 months’ anticoagulation</td>
<td>31.6 (20.0–46.0)</td>
</tr>
<tr>
<td></td>
<td>6.0 (3.9–10.5)</td>
</tr>
<tr>
<td></td>
<td>0.42 (0.35–0.49)</td>
</tr>
<tr>
<td>p value</td>
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</tr>
<tr>
<td></td>
<td>0.232</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001</td>
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</table>

\textsuperscript{a}sEsel: soluble E-selectin.

\textsuperscript{b}IL-6: interleukin 6.

\textsuperscript{c}F1+2: prothrombin fragment 1+2.
the presence of abnormal thrombogenesis (high F1+2 levels) in AF. Furthermore, no changes in IL-6 levels were found despite the reduction of the other markers by introducing anticoagulant therapy.

Acknowledgements

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