

## Theme Issue Article

# Circulating endothelial cells in atrial fibrillation with and without acute cardiovascular disease

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### Summary

Normal adults have very few circulating endothelial cells (CECs) in their blood, but increased levels have been shown in association with conditions associated with endothelial damage such as myocardial infarction and stroke. As atrial fibrillation (AF) is associated with a hypercoagulable state and abnormalities of plasma indices of endothelial damage/dysfunction, we hypothesised that CECs would also be raised in this condition, and would correlate with these plasma markers. We measured CECs (by immunofluorescence) as an indicator of frank endothelial damage, alongside 3 plasma indices of endothelial perturbation: von Willebrand factor (vWf), soluble E-selectin and soluble thrombomodulin (sTM) (all ELISA) in 28 patients with chronic 'stable' AF, 63 patients with AF plus an acute cardiovascular or cerebrovascular event as positive controls, and 20 healthy sub-

jects in sinus rhythm as negative controls. Chronic 'stable' AF patients had significantly higher levels of plasma vWf ( $p < 0.001$ ), but comparable numbers of CECs ( $p = 0.1638$ ) in comparison to healthy controls. In patients with AF associated with an acute cardiovascular or cerebrovascular event, levels of CECs ( $p < 0.0001$ ) and sTM ( $p = 0.004$ ), but not vWf or sEsel, were significantly increased in comparison to chronic 'stable' AF patients. Patients with uncomplicated AF have abnormal systemic endothelial damage/dysfunction, as evident by increased plasma vWf levels, but normal numbers of CECs, compared to subjects in sinus rhythm. However, following clinical complications, such as stroke or significant haemodynamic compromise, further endothelial disturbance (as indicated by high levels of sTM and CECs) suggests additional endothelial damage.

### Keywords

Circulating endothelial cells, endothelial damage, soluble E-selectin, soluble thrombomodulin, von Willebrand factor, atrial fibrillation

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## Background

Atrial fibrillation (AF) is the most common non-rheumatic cardiac arrhythmia, is associated with both haemodynamic and thromboembolic complications, and is therefore a major cause of morbidity and mortality. As the population ages, increased numbers of subjects with this condition pose a serious and growing Public Health issue. For example, AF is not only associated with a five-fold increase in risk of stroke, but is also associated with worse deficits and worse outcomes than for stroke patients in sinus rhythm (1, 2). Similarly, AF associated with acute myocardial infarction is related to unfavourable outcomes (3, 4), and AF in patients with cardiac failure is again associated with a worse prognosis (5). Consequently, the pathophysiology of this condition demands investigation.

Endothelial damage/dysfunction has been frequently described in 'steady state' chronic AF with abnormal plasma indices of endothelial perturbation, such as von Willebrand factor (vWf), soluble E-selectin and soluble thrombomodulin (sTM) (6–11). Circulating endothelial cells (CECs) provide perhaps the most direct evidence of endothelial injury, with increased numbers seen in conditions associated with generalised endothelial damage, such as sickle cell crisis, inflammatory vasculitis and septic shock (12–14). Numbers of CECs can also be raised in coronary angioplasty, and in cardiovascular and lung disease, such as acute myocardial infarction, critical limb ischaemia, acute stroke and pulmonary hypertension (15–19). Increased numbers of CECs appear not to be a feature of generalised atherosclerosis per se, as levels are near-normal in stable angina, stable peripheral vascular disease (intermittent claudication) pa-

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tients and systemic hypertension (16, 17). However, we are not aware of previous studies of CECs in patients with AF.

We first hypothesised that, compared to healthy age and sex matched controls, increased numbers of CECs are present in AF, and are related to levels of more established plasma markers of endothelial perturbation (vWf, soluble E-selectin, and sTM), as further evidence of endothelial damage/dysfunction in AF. We secondly hypothesised that numbers of CECs would be higher in AF patients suffering an acute cardiovascular or cerebrovascular event, compared to stable AF patients, possibly reflecting the more severe and life-threatening disease in the former.

## Methods

### Subjects

We recruited 28 patients with chronic 'stable' AF (no hospital admission in the prior 3 months) from Routine Out-Patient clinics, and 63 AF patients with an acute cardiovascular or cerebrovascular event. The latter were recruited within 48 hours of hospital admission and had electrocardiographic evidence of AF at the time of presentation. Acute myocardial infarction (MI) was defined as the presence of typical sustained chest pain, electrocardiogram (ECG) changes typical of acute MI, and raised peak creatine kinase within 24 hours after the onset of symptoms according to established criteria of the World Health Organization (median peak total creatine kinase rise 844 IU/ml (inter-quartile range 307–1974)). Two patients on warfarin who went for primary angioplasty were excluded from the analysis, as CEC count has been shown to be raised following elective intravascular interventions (15). Acute heart failure patients had to have radiographic evidence of pulmonary oedema as well as clinical evidence of heart failure on presentation, and had documented left ventricular ejection fraction of  $\leq 40\%$  either by M-mode echocardiography or by Simpson's method (where there was the presence of significant regional wall motion abnormality). Mean (standard deviation) left ventricular ejection fraction for the heart failure patients was 27.0 (10.4)%. Acute stroke patients all had a focal neurological deficit at presentation persisting for  $> 24$  hours with cerebral infarction confirmed on CT scan report during admission. Cerebral haemorrhage was excluded.

Twenty healthy control subjects were recruited from amongst healthy hospital staff and from relatives or friends of patients. They had no clinical evidence of vascular, metabolic, neoplastic, diabetic or inflammatory disease on careful history, examination and routine laboratory tests. For all subjects, evidence of concomitant infection or pyrexial illness at presentation, or a history of chronic or systemic illnesses (including renal failure, hepatic impairment, cancer, inflammatory connective tissue disease and inflammatory bowel disease) were exclusion criteria. The study protocol was approved by the West Birmingham Research Ethics Committee and all patients (or in some stroke patients, next of kin) gave informed consent before a blood sample was taken.

### Laboratory

Citrated plasma was obtained from venous blood by centrifugation at 3000 rpm (1000 g) for 20 min at 4°C. Aliquots of citrated plasma were stored at  $-70^{\circ}\text{C}$  to allow batch analysis. vWf was measured by an established ELISA (Dako, Ely, UK). Sol-

uble E-selectin was measured by ELISA with R&D Systems reagents (Abingdon, United Kingdom). sTM was also measured by ELISA (kits from Diagnostica Stago, France). The intra-assay coefficient of assays was  $< 5\%$ , inter-assay variation was  $< 10\%$ .

Estimation of CECs has been described fully elsewhere (13, 15–19). In brief, 4 ml of blood collected in vacutainer tubes was mixed with 4 ml of normal saline. To this mixture, 100 ml of a suspension of monodispersed magnetic 4.5 mm diameter polystyrene beads (Dynabeads M-450, Dynal A.S., Oslo, Norway) coated with a secondary layer of S-Endo 1 (a monoclonal antibody [sEndo-1] recognising endothelial specific CD146 [Biocytex, Marseille, France]) was added. This mixture was incubated at room temperature for 30 min whilst being gently rotated (30 rpm) to ensure continued mixing. The rosetted beads were separated from the blood using an MPC-L concentrator (Dynal) and washed a total of four times. The resulting rosetted cells and beads were finally re-suspended in  $\sim 30$  ml of PBS and dispersed on a glass slide for counting by a single observer under epifluorescence microscopy (Zeiss, Welwyn Garden City, UK). CECs are easily located as they are autofluorescent although there is considerable fluorescent debris. The latter is easily identified by non-cellular morphology and intense fluorescence. The criteria for confirmation of a CEC was binding  $\geq 4$  beads and a clear and regular cell morphology of greater than 20  $\mu\text{m}$  diameter (approximately four times bead diameters), or 10 beads with an irregular morphology. For aggregated cells, the aggregate was counted as a single cell. Intra- (n=40) and inter-assay (n=20) coefficients of variation were  $< 5\%$  and  $< 10\%$  respectively. The inter- and intra-observer variations of the method in our laboratory were  $< 5\%$  (n=20 determinations). All laboratory work was performed in a blinded fashion with respect to the identity of the samples.

### Power calculations and statistical analysis

Increased CECs in the plasma of 26 subjects with acute myocardial infarction (AMI) and 33 with unstable angina have been previously reported and compared to 13 with stable angina and 14 healthy controls with an overall F statistic of 16 giving a p value of  $< 0.001$  (16). Others have published CEC data with groups of 12–20 subjects (13), 29–30 subjects (18), or 12–14 patients (19). We therefore hypothesised similar levels and distribution in AF patients with 'acute events' compared to chronic stable AF. Our power calculation required 20 subjects per group to generate a similar F statistic at  $p < 0.001$ . This target number of subjects also provides the power to detect a correlation coefficient of 0.35 at  $p < 0.05$  and  $1 - \beta = 0.85$ .

Data were analysed by the Shapiro-Wilks test to determine distribution. Normally distributed data are expressed as mean and standard deviation. As the data for soluble E-selectin, sTM and CECs were not normally distributed, values were expressed as median (interquartile range, IQR). Baseline cross-sectional data between acute events, chronic AF and healthy controls were analysed by ANOVA, Mann-Whitney or Kruskal-Wallis test as appropriate, with between group comparisons by Tukey's post hoc test and, if appropriate, after log transformation. Categorical data were compared using Chi-squared test. Correlations were performed using Spearman's rank correlation method. Multivariate analysis was performed by stepwise multiple regression

**Table 1: Clinical variables and endothelial markers for chronic atrial fibrillation in comparison to controls.**

|                           | Healthy controls (n=20) | Chronic AF (n=28) | p value |
|---------------------------|-------------------------|-------------------|---------|
| <b>Clinical measures</b>  |                         |                   |         |
| Mean age (years)          | 64 ± 9                  | 66 ± 5            | 0.310   |
| Males, n (%)              | 8 (40%)                 | 19 (68%)          | 0.055   |
| Current smokers, n (%)    | 1 (5%)                  | 1 (4%)            | 0.801   |
| Mean systolic BP (mmHg)   | 129 ± 17                | 137 ± 20          | 0.165   |
| Mean diastolic BP (mmHg)  | 76 ± 8                  | 82 ± 14           | 0.074   |
| Total Cholesterol (mM/dl) | 5.7 ± 1.0               | 4.5 ± 1.0         | 0.001   |
| <b>Research indices</b>   |                         |                   |         |
| vWf (iU/dl)               | 100 ± 51                | 169 ± 42          | <0.0001 |
| sE-selectin (ng/ml)       | 28 (20–55)              | 52 (23–93)        | 0.161   |
| sTM (ng/ml)               | 36 (25–49)              | 38 (18.5–53.5)    | 0.9416  |
| CEC (cells/ml)            | 4.5 (1.6–7.2)           | 5.25 (3.25–9.75)  | 0.1638  |

BP= blood pressure, vWf = von Willebrand factor, sE-sel= soluble E-selectin, sTM=soluble thrombomodulin, CEC= circulating endothelial cells. Values are given as number (percentage), mean values ± standard deviation, or median values (interquartile range).

analysis using CEC as the dependent variable and clinical variables (e.g. age, gender, hypertension, coronary artery disease, etc.) and the presence/absence of AF as predictors. A two-tailed p value <0.05 was considered statistically significant.

**Results**

Clinical and demographic data on the subjects are presented in tables 1 and 2. Our first hypothesis was of raised CECs in AF compared to sinus rhythm. As previously demonstrated (6–9), vWf levels were significantly higher in AF than in controls but there was no significant difference in numbers of CECs (Fig. 1) or levels of soluble E selectin or sTM. We ascribe lower cholesterol levels in the patients to the use of statins.

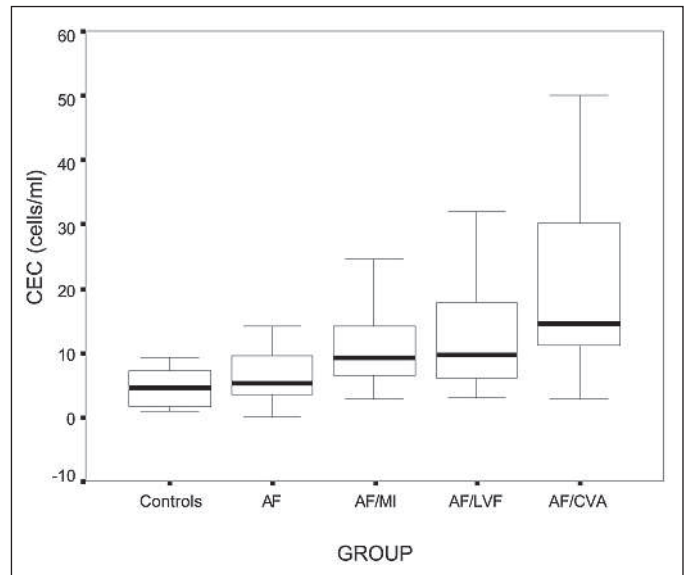
Our second hypothesis predicted higher numbers of CECs in patients with AF plus an acute vascular event compared to patients with stable AF (Table 3). CEC counts were raised in all acute complication subgroups, in comparison to stable AF alone (Fig. 1). Plasma vWf levels, which were already raised in stable AF in comparison to healthy controls, were not significantly elevated further by an additional acute event. Plasma soluble E-selectin levels were no different between stable AF and AF with acute complications. Plasma sTM levels, although not raised in stable AF alone, were significantly raised in association with each acute syndrome (MI, LVF and CVA).

Analysis of all AF patients showed that CEC counts correlated with plasma levels of vWf, although this was poor (Spearman, r=0.307, p=0.004) (Fig. 2). Correlations with soluble E selectin and sTM were not significant. On univariate analysis of AF patients and subgroups, CECs were significantly correlated with clinical subgroup (i.e. MI, LVF, stroke) (p<0.0001) and smoking (p=0.03), but on stepwise multiple regression analyses, only clinical subgroup was an independent predictor of CEC levels (p<0.0001).

**Table 2: Clinical data for patient groups.**

|                                | Chronic AF | MI/AF     | LVF/AF    | CVA/AF    | p value |
|--------------------------------|------------|-----------|-----------|-----------|---------|
| n                              | 28         | 22        | 20        | 21        |         |
| Mean Age (yrs)                 | 66 ± 5     | 67 ± 10   | 70 ± 9    | 71 ± 7    | 0.081   |
| Male, n (%)                    | 19 (68%)   | 15 (68%)  | 13 (65%)  | 13 (62%)  | 0.133   |
| Smokers, n (%)                 | 1 (4%)     | 5 (23%)   | 4 (20%)   | 4 (19%)   | 0.017   |
| Mean SBP (mmHg)                | 137±20     | 130±30    | 113±20    | 141±19    | <0.0001 |
| Mean DBP (mmHg)                | 82±14      | 70±16     | 66±13     | 79±12     | 0.001   |
| Total Chol (mMol/dl)           | 4.5 ± 1.0  | 5.1 ± 1.1 | 4.3 ± 1.1 | 4.6 ± 1.4 | 0.163   |
| Median duration AF (months)    | 6 (4–8)    | 0(0–6)    | 12(1–36)  | 18(0–26)  | 0.003   |
| <b>Past medical history</b>    |            |           |           |           |         |
| Hypertension                   | 11 (37%)   | 14 (54%)  | 7 (32%)   | 13 (62%)  | ...     |
| Diabetes Mellitus              | 1 (3%)     | 9 (35%)   | 3 (15%)   | 3 (13%)   | ...     |
| Angina/MI                      | 3 (10%)    | 8 (31%)   | 10 (50%)  | 0         | ...     |
| <b>Concomitant medications</b> |            |           |           |           |         |
| Aspirin                        | 9 (32%)    | 22 (100%) | 4 (18%)   | 7 (33%)   | ...     |
| Heparin                        | 0          | 11 (50%)  | 0         | 0         | ...     |
| Warfarin                       | 28 (100%)  | 4 (18%)   | 17 (85%)  | 8 (38%)   | ...     |
| BB                             | 8 (29%)    | 15 (68%)  | 2 (10%)   | 3 (14%)   | ...     |
| CCB                            | 7 (25%)    | 4 (18%)   | 1 (5%)    | 3 (14%)   | ...     |
| ACEi/ARB                       | 13 (46%)   | 12 (55%)  | 11 (55%)  | 5 (24%)   | ...     |
| Statin                         | 4 (14%)    | 17 (77%)  | 7 (35%)   | 5 (24%)   | ...     |
| Digoxin                        | 6 (21%)    | 5 (23%)   | 15 (75%)  | 7 (33%)   | ...     |
| Amiodarone                     | 1 (4%)     | 3 (14%)   | 5 (25%)   | 0         | ...     |
| Nitrate                        | 0          | 5 (23%)   | 5 (25%)   | 0         | ...     |

All values are given as number and percentage unless otherwise indicated. For mean values the standard deviation and for median values the interquartile range is also given. AF= atrial fibrillation; MI= myocardial infarction; CVA= cerebrovascular accident; LVF= left ventricular failure; SBP= systolic blood pressure; DBP= diastolic blood pressure; Chol= cholesterol; BB=beta-blocker; CCB= calcium channel blocker; ACEi= angiotensin converting enzyme inhibitor; ARB=angiotensin receptor blocker.

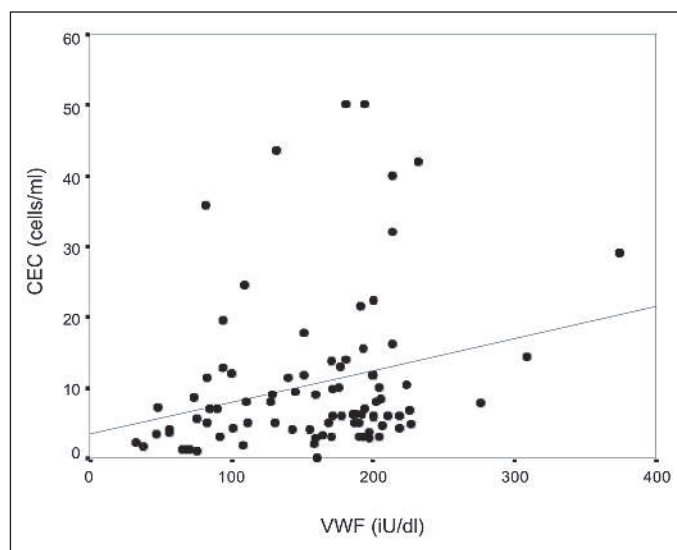


**Figure 1: CEC levels in different patient groups and healthy controls.**

**Table 3: Results of research indices for patients.**

|                | AF<br>n=28 | AF/MI<br>n=22 | AF/LVF<br>n=20 | AF/CVA<br>n=21          | p value           |
|----------------|------------|---------------|----------------|-------------------------|-------------------|
| vWf (iU/dl)    | 169 ± 42   | 194 ± 28      | 186 ± 80       | 164 ± 52                | 0.437             |
| sE-sel (ng/ml) | 52 (23–93) | 42 (29–54)    | 50 (27–83)     | 35 (22–50)              | 0.292             |
| sTM (ng/ml)    | 38 (19–54) | 50 (46–118)*  | 50 (35–63)**   | 48 (43–63) <sup>†</sup> | <b>0.004</b>      |
| CEC (cells/ml) | 5 (3–10)   | 9 (6–15)      | 10 (6–19)      | 15 (11–33)              | <b>&lt;0.0001</b> |

vWf = von Willebrand factor, sE-sel = soluble E-selectin, sTM = soluble thrombomodulin, CEC = circulating endothelial cells. Levels are expressed as mean ± standard deviation, except sE-sel, sTM and CEC which are expressed as median (inter-quartile range). Analysis was by ANOVA and Kruskal-Wallis respectively. For sTM, \*p=0.0103 \*\*p=0.0466 and <sup>†</sup>p=0.0266 on Mann-Whitney testing between acute event and chronic AF patients.

**Figure 2: Correlation of vWf with CECs.**

## Discussion

CECs are raised in association with many conditions and are taken to be evidence of direct endothelial injury (18, 20). We now add to this in demonstrating raised CEC numbers in patients with an acute cardiovascular or cerebrovascular event complicated by AF, but no change in patients with uncomplicated ‘stable’ AF. Thus, we suggest that the established endothelial perturbation in this arrhythmia (as demonstrated by raised vWf levels) is of insufficient severity to a severe vascular insult. Plasma vWf levels, which are already known to be elevated in AF (shown in both this and numerous previous studies) (6–9), are also high in AF associated with an acute event, but levels do not appear to be significantly higher than the levels already seen in chronic stable AF patients. Neither soluble E-selectin, marking ‘inflammatory’ endothelial activation (21), nor sTM, implying changes in cell-surface haemostasis, were raised in stable AF, suggesting they reflect different aspects of vascular pathophysiology. By contrast, in AF patients with and without an acute vascular event, both sTM and CECs were raised in more severe disease and probably imply a different ‘level’ of endothelial perturbation in these conditions.

In line with our previous work in peripheral vascular disease (17), we have again shown CEC levels to correlate with vWf levels, further emphasising the importance of vWf and CECs as *in vivo* evidence of endothelial dysfunction and damage respectively. Indeed, the most plausible explanation of these findings is of a background of endothelial dysfunction in ‘steady-state’ stable AF, as indicated by raised vWf levels, and raised CEC and sTM levels in acute complications associated with AF (perhaps a measure of ‘target organ damage’) being representative of actual damage to an already fragile endothelium. CEC levels in AF appear comparable to healthy controls on this analysis, and are only elevated with ‘target organ damage’ or heart failure, rather than the risk factor *per se*. This is again consistent with previous findings of raised CEC levels in MI compared with angina, or in critical limb ischaemia compared with peripheral vascular disease, and is taken in this case to indicate actual vessel wall damage (16, 17).

In the case of acute MI, mechanical detachment during plaque rupture has been hypothesised (16), but there are several alternative possibilities as to the mechanism of detachment. One possibility is ischaemic endothelial damage, as hypoxia has been demonstrated to cause cell detachment, at least in an *in vitro* model (22), and alterations in flow conditions have also been shown to cause endothelial disruption (23). Thus, increases in CECs could originate from the vasculature following upstream thrombotic or thromboembolic occlusion in the case of MI or CVA, and peripheral ischaemia during acute heart failure could also be hypothesised to lead to release of endothelial cells from the microcirculation. Another common link could be endothelial cell detachment via the increases in pro-inflammatory cytokines reported in MI, CVA and heart failure (25–27). Indeed, interleukins and tumour necrosis factor could perpetuate endothelial damage in the setting of the already dysfunctional endothelium seen in AF, as some cytokines have been shown to cause cell detachment, again at least *in vitro* (28).

We are also aware of the debate as to whether or not CECs could in fact be progenitor cells originating from the bone marrow. Our method uses an immunomagnetic separation assay based on the S-Endo 1 monoclonal antibody directed against the endothelial antigen CD146 which is present on mature endothelial cells but not haematopoietic cells (29). In addition, our studies have previously shown that CD146-immunobead defined CECs were positive for CD31 but negative for CD34, and therefore these cells are unlikely to be progenitor cells (that have been shown to bear CD34 but not CD31) (16). Further to this, the association of CECs with other endothelial plasma markers would be in support of these cells being shed from the vascular endothelium. Of note, a previous study by Lin et al. (30) supports the vascular origin of CECs, as they found that in gender-mismatched bone marrow transplant recipients, most CEC in fresh blood had the recipient genotype, therefore concluding that CEC found in the recipient blood were derived from the recipient vessel wall rather than the donor bone-marrow. We are also aware of the debate as to whether or not CECs are apoptotic or non-apoptotic, but would argue that in either case, detached endothelial cells could act as a potent pro-coagulant stimulus, with the potential for release of prothrombotic substances such as tissue factor and into the circulation (20).

Our study is, of course, limited by its cross-sectional design and therefore only associations can be noted, but would imply that endothelial damage occurs in AF at the time of an acute event. Nonetheless, the observed increases in sTM and CECs in association with already raised vWf in AF patients in the situation of an acute event may heighten the prothrombotic environment, with implications for worsening prognosis in these patients, due to a predisposition to further thrombotic events. We accept that the influence of CECs and endothelial damage on hypercoagulability is difficult to gauge but merely postulated (20). Certainly for this study, as plasma levels of coagulation components and fibrinolytic products would be inaccurate in the presence of therapeutic thrombolytic agents, anti-thrombotic treat-

ment and anticoagulants, these were not measured. We also note that published levels vWf and CEC in controls have a degree of variability between published studies, which may reflect different study populations and methodology/settings (17, 20, 31). Ongoing work in our department is currently defining the influence of demographic features as well as refining the definition/detection of CECs.

In conclusion, severe endothelial damage, as assessed by increased numbers of CECs, appears not to be a prominent feature of chronic AF, but levels are certainly raised in AF at the time of an acute vascular complication. We speculate that this contributes to an enhanced pro-thrombotic state.

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