Platelet Expression of CD40/CD40 Ligand and Its Relation to Inflammatory Markers and Adhesion Molecules in Patients with Atrial Fibrillation

Matthias Hammwöhner,* Annelore Ittenson,† Jutta Dierkes,‡ Alicja Bukowska,§ Helmut U. Klein,* Uwe Lendeckel,§ and Andreas Goette*,[1]

*Division of Cardiology, Department of Internal Medicine, †Institute of Immunology, ‡Department of Laboratory Medicine, and §Institute of Experimental Internal Medicine, University Hospital Magdeburg, Magdeburg 39120, Germany

Recent studies suggest the importance of prothrombotic and proinflammatory cascades in vascular thrombus formation. However, the impact of platelet CD40 and CD40 ligand (CD40L) expression and its relation to inflammatory markers in atrial clot formation have not yet been determined. Therefore, we studied a total of 40 patients. A total of 20 patients with persistent atrial fibrillation (AF) and 20 matched patients with sinus rhythm (SR) were included to quantify platelet surface expression of CD40/CD40L, serum levels of intercellular adhesion molecule-1 (ICAM), vascular adhesion molecule-1 (VCAM), high-sensitivity C-reactive protein (hsCRP), and monocyte chemoattractant protein-1 (MCP-1). Using fluorescence-activated cell sorting analysis, baseline CD40 expression (antibody binding capacity [ABC]) was increased during AF (AF: 7776 ± 8.46 ABC vs. SR: 7753 ± 7.32 ABC; $P < 0.05$), whereas CD40L expression was not different. In contrast to the effect of adenosine diphosphate, *ex vivo* stimulation with thrombin receptor activating peptide (TRAP) increased CD40 and CD40L expression in both groups. MCP-1, hsCRP, ICAM, and VCAM levels were significantly increased during AF, reaching highest levels in patients with atrial thrombi. Importantly, VCAM and MCP-1 were independent predictors for atrial thrombi ($P < 0.05$) using multivariate analysis. In contrast to declining levels of hsCRP, levels of ICAM, VCAM, MCP-1, and platelet CD40 expression remained elevated 5 weeks after successful electrical direct current cardioversion (CV). In conclusion, prothrombogenic markers are substantially elevated in patients with AF, reaching highest levels in patients with AF and atrial thrombi. Interestingly, amounts of adhesion molecules and platelet CD40 levels remain elevated even 5 weeks after successful CV, which may imply a persistently increased risk for atrial thrombus formation. In addition to hsCRP, MCP-1 and VCAM may serve as new biomarkers, which may help to identify patients with an increased risk for thromboembolic events. Exp Biol Med 232:581–589, 2007

Key words: adhesion molecules; CD40; fibrillation; inflammation; remodeling; platelets

Introduction

Several studies have shown that atrial fibrillation (AF) is accompanied by a hypercoagulative state that may contribute to the development of atrial thrombi and thromboembolism (1–9). In addition to enhanced activation of the plasma coagulation system, AF also influences platelet aggregability. Sohara *et al.* showed an acceleration of platelet activity developing 12 hrs after the onset of AF (6). Increasing evidence has emerged that activated platelets play a pivotal role in inflammatory processes as a result of platelet–leukocyte and platelet–endothelium interactions; thus, platelets are important and abundant inflammatory cells *per se* (10–12).

There also is increasing evidence of the importance of cellular adhesion molecule monocyte chemoattractant protein-1 (MCP-1) and CD40/CD40 ligand (CD40L) interaction among platelets and endothelial cells, where they trigger, modulate, or abate inflammatory mechanisms, especially in patients with acute coronary syndromes (13–17).

In the setting of persistent AF, a marked increase in levels of inflammatory markers such as high-sensitivity C-reactive protein (hsCRP) and interleukin-6 (IL-6) has been

This work was supported by a grant from the Bundesministerium für Bildung und Forschung, Germany (Kompetenznetz Vorhofflimmern, 01GI0204).

[1] To whom correspondence should be addressed at Division of Cardiology, Department of Internal Medicine, University Hospital Magdeburg, Leipzigerstr. 44, 39120 Magdeburg, Germany. E-mail: andreas.goette@medizin.uni-magdeburg.de

Received July 18, 2006.
Accepted November 24, 2006.

1535-3702/07/2324-0581$15.00

Table 1. Baseline Patient Characteristics[a]

	Controls ($n = 20$)	AF ($n = 20$)	P
Age, years	60.05 ± 3.22	63.35 ± 2.25	—
Male/female, *n*	12/8	13/7	—
Weight, kg	84.3 ± 3.61	91.85 ± 3.42	—
BMI, kg/m^2	28.75 ± 1.25	30.4 ± 1.21	—
SBP, mm Hg	148.13 ± 6.01	133.32 ± 4.66	—
DBP, mm Hg	87.19 ± 2.92	81.16 ± 4.75	—
Current NYHA class	0.93 ± 0.31	1.37 ± 0.26	—
LVEF, %	55.59 ± 1.86	48.75 ± 3.66	—
LAD(s), cm	3.99 ± 0.18	4.78 ± 0.14	<0.01
LVEDD, cm	5.07 ± 0.34	5.14 ± 0.39	—
Diabetes, *n* (%)	2 (10)	6 (30)	—
Hypertension, *n* (%)	12 (70)	17 (85)	—
Left ventricular hypertrophy, *n* (%)	9 (45)	10 (50)	—
History of CAD, *n* (%)	3 (15)	3 (15)	—
Previous myocardial infarction, *n* (%)	1 (5)	1 (5)	—
Previous CABG/PTCA, *n* (%)	1 (5)	1 (5)	—
Valvular heart disease, *n* (%)	0 (0)	2 (10)	—
Previous thromboembolism/stroke, *n* (%)	0 (0)	2 (10)	—
Left atrial thrombus, *n* (%)	0 (0)	6 (30)	<0.001
History of smoking, *n* (%)	7 (35)	9 (45)	—
Current smoking, *n* (%)	3 (15)	2 (10)	—
Hyperlipoproteinemia, *n* (%)	10 (50)	14 (70)	—
Class I antiarrhythmic drugs, *n* (%)	0 (0)	2 (10)	—
Class II antiarrhythmic drugs, *n* (%)	4 (20)	16 (80)	<0.001
Class III antiarrhythmic drugs, *n* (%)	0 (0)	6 (30)	<0.05
Class IV antiarrythmic drugs, *n* (%)	0 (0)	1 (5)	—
Coumadin, *n* (%)	0 (0)	14 (70)	<0.05
ACE inhibitors/AT1 antagonists	8 (40)	16 (80)	<0.05
Dihydropyridine calcium antagonists, *n* (%)	1 (5)	2 (10)	—
Statins, *n* (%)	2 (10)	4 (20)	—

[a] Values are given as means ± SEM or *n* (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; CAD, coronary artery disease; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty; ASA, acetyl salicylic acid; ACE, angiotensin converting enzyme; AT1, angiotensin-II type 1 receptor.

identified (18, 19). However, the relationship between proinflammatory and prothrombogenic factors remains unclear, and platelet expression of CD40 and CD40L has not yet been assessed in patients with AF. Therefore, the purpose of the present study was to determine platelet surface expression of CD40 and CD40L before and after electrical direct current cardioversion (CV) of persistent AF. Furthermore, platelet activity was determined before and after *ex vivo* stimulation with thrombin receptor activating peptide (TRAP) and adenosine diphosphate (ADP). Activation of the CD40/CD40L system was correlated with systemic levels of intercellular adhesion molecule-1 (ICAM), vascular adhesion molecule-1 (VCAM), hsCRP, and MCP-1 in patients with and without persistent AF.

Materials and Methods

Patient Characteristics. A total of 40 patients were included in our prospective study. A total of 20 patients with persistent AF (duration $\geq$ 4 months) were compared with 20 age- and gender-matched control patients in sinus rhythm (SR). Matching with regard to age was done within a limit of a 5-year difference. Detailed patient characteristics and medication used are listed in Table 1. In all patients, a 12-lead electrocardiogram and an echocardiogram were carried out at baseline. The study was approved by the Ethics Committee of the University Hospital Magdeburg, Germany, and all patients gave written informed consent to participate in the study.

Asservation of Blood Samples. After 30 mins in resting position, 22 ml blood was drawn from a cubital vein using an 18-gauge needle (Becton Dickinson, Madrid, Spain) without prior tourniquet application. Three 5-ml standard sodium citrate tubes (0.105 *M* sodium citrate) and one 7-ml standard serum tube were used for each subject. Two of the sodium citrate tubes and the serum tube were immediately cooled down to 4°C. The other citrate tube remained at room temperature. All tubes were immediately referred for further processing and analysis.

Platelet CD40/CD40L Expression. Blood samples collected in tubes containing 0.105 *M* sodium citrate were used for analysis of platelet CD40/CD40L expression. Platelet counts were determined by CELL-DYN 1600 Counter (Abbott, Wiesbaden, Germany). Platelets were diluted to 20×10^9/l with buffered Hanks' balanced salt

solution + 1 mg/ml bovine serum albumin (BSA). A 36-µl volume of diluted blood was incubated for 10 mins at 37°C in: (i) a tube containing 4 µl buffer, (ii) a tube containing 4 µl of 50 µ*M* ADP, and (iii) a tube containing 4 µl of 100 µ*M* TRAP. For platelet identification, each sample was further incubated with 20 µl phycoerythrin (PE)–labeled monoclonal antibody against platelet glycoprotein IIb/IIIa (CD41a; Beckmann Coulter, Krefeld, Germany) and then with fluorescein isothiocyanate–labeled P-selectin (CD62P; Beckmann Coulter), as well as with monoclonal antibody against CD40 (FA CALTAG Laboratories; Karlsruhe, Germany) and CD40L (FA Bender MedSystems; Vienna, Austria) for 5 mins at 37°C. The process of activation and staining was stopped in each aliquot with 2 ml cold buffer (Hanks' balanced salt solution, 4°C). The platelet fluorescence was determined by flow cytometry using a fluorescence-activated cell sorter (FACS) Calibur (Becton Dickinson, Heidelberg, Germany; Ref. 20). The quantification of the various antibody binding sites (CD62P, CD40, and CD40L) was performed by analysis of the fluorescence intensity using a Quantum Simply Cellular Microbeads Kit (Bangs Laboratories, Fishers, IN). After internal calibration, data were presented as antibody binding capacity (ABC).

Systemic Levels of hsCRP, ICAM, VCAM, and MCP-1. Levels of hsCRP were determined using a random access analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany) and CRP Dynamic Reagenz (BIOMED Labordiagnostik GmbH, Oberschleißheim, Germany); this is a latex particle-enhanced immunoturbidimetric assay. The range of the assay is from 0–30 mg/l. ICAM, VCAM, and MCP-1 levels were measured using a quantitative sandwich enzyme immunoassay technique (R&D Systems, Wiesbaden, Germany). The coefficients of variation of these assays were 4.5%, 6.1%, and 3.8%, respectively.

Electrical CV. In patients with AF (six patients were excluded from CV due to atrial thrombi), direct current external CV was performed using a single monophasic 360-J shock under anesthetic with body weight–adapted doses of midazolam and etomidate. In 10 patients, who thereafter remained in stable SR, another set of blood samples was taken, as described above, at 24 and 48 hrs after CV and 5 weeks after CV.

Statistical Analysis. Data are expressed as means ± SEM. Intergroup and intragroup differences were analyzed by ANOVA. The chi-square test, univariance analysis, and multinominal logistic regression analyses were used, where appropriate, to assess the association between the occurrence of AF, biomarkers (ICAM, VCAM, MCP-1, hsCRP) and clinical parameters (age, diabetes, New York Heart Association class, hypertension, smoking, left atrial diameter, atrial thrombus). The Pearson coefficient (r) was used to determine correlations between the various metric parameters. All statistical data were calculated using SPSS (SPSS Inc., Chicago, IL). A P value < 0.05 was considered to be statistically significant.

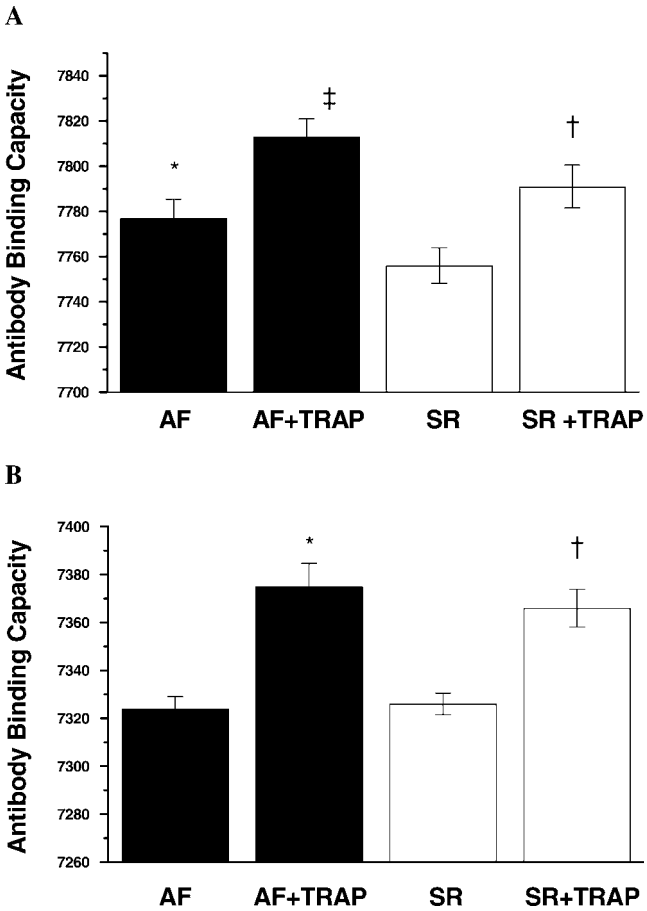


Figure 1. (A) Platelet CD40 expression before and after *ex vivo* TRAP stimulation in patients with AF (black columns) and SR (white columns). Values are means ± SEM. *$P < 0.05$ baseline AF vs. baseline SR; †$P < 0.01$ baseline SR vs. SR after TRAP stimulation; ‡$P < 0.0001$ baseline AF vs. AF after TRAP stimulation; $P = 0.07$ AF after TRAP stimulation vs. SR after TRAP stimulation. (B) Platelet CD40L expression before and after *ex vivo* TRAP stimulation in patients with AF (black columns) and SR (white columns). Values are means ± SEM. *$P < 0.001$ baseline AF vs. AF after TRAP stimulation; †$P < 0.01$ baseline SR vs. SR after TRAP stimulation.

Results

Platelet CD40/CD40L Expression. Platelet CD40 expression was increased during AF compared with patients with SR (AF: 7776 ± 8.46 ABC vs. SR: 7753 ± 7.32 ABC; $P < 0.05$), whereas baseline platelet CD40L levels were comparable in the two groups (AF: 7324 ± 5.03 ABC vs. SR: 7326 ± 4.50 ABC; P = ns). CD40/CD40L expression did not correlate with clinical variables such as age, gender, duration of AF, concomitant diseases, and medication (data not shown).

Ex vivo stimulation with TRAP induced a substantial increase in CD40 expression in both the AF and SR groups (AF: 7776 ± 8.46 ABC vs. 7813.7 ± 7.98 ABC, $P < 0.001$; SR: 7753 ± 7.32 ABC vs. 7791 ± 9.44 ABC, $P < 0.01$). CD40 expression remained higher in the AF group compared with the SR group after TRAP stimulation (Fig. 1A). *Ex vivo* TRAP stimulation also increased CD40L

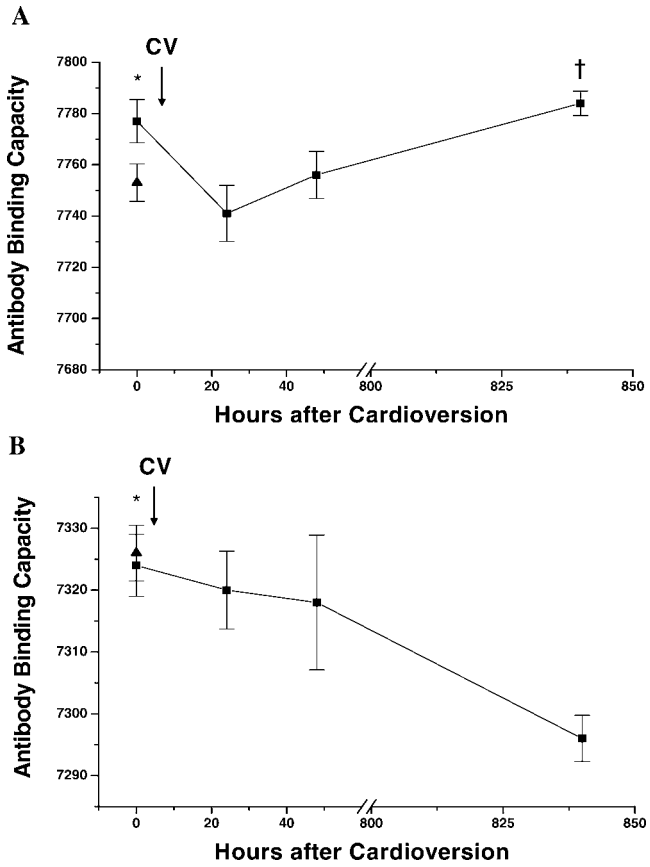


Figure 2. (A) Platelet CD40 expression before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (square); SR (triangle). Values are means ± SEM. *$P < 0.05$ AF before CV vs. baseline SR; †$P < 0.01$ AF after CV vs. baseline SR. (B) Platelet CD40L expression before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (square); SR (triangle). Values are means ± SEM. *$P < 0.05$ AF before CV vs. AF 5 weeks after CV.

expression in both the AF and SR groups (AF: 7324 ± 5.03 ABC vs. 7376 ± 9.66 ABC, $P < 0.01$; SR: 7326 ± 4.50 ABC vs. 7366 ± 7.91 ABC, $P < 0.001$), with no difference between the two groups after stimulation (Fig. 1B). ADP stimulation did not show a significant effect on CD40 (AF: 7776 ± 8.46 ABC vs. 7774 ± 10.76 ABC, $P =$ ns; SR: 7753 ± 7.32 ABC vs. 7771 ± 8.81 ABC, $P =$ ns) or CD40L expression (AF: 7324 ± 5.03 ABC vs. 7330 ± 9.66 ABC, $P =$ ns; SR: 7326 ± 4.50 ABC vs. 7328 ± 4.47 ABC, $P =$ ns).

As shown previously (9), TRAP stimulation caused a significant increase in platelet P-selectin (CD62P) in the AF group (AF: 7779 ± 23.41 ABC vs. 121131 ± 547.62 ABC, $P < 0.000001$; SR: 7740 ± 23.49 ABC vs. 10245 ± 413.75 ABC, $P < 0.00001$).

CD40 expression remained elevated after successful CV (before CV: 7776 ± 8.46 ABC; 24 hrs after CV: 7745 ± 36.22 ABC; 48 hrs after CV: 7760 ± 24.4 ABC; 5 weeks after CV: 7784 ± 9.56 ABC; $P =$ ns; Fig. 2A). In contrast, CD40L levels declined progressively after successful CV (before CV: 7324 ± 5.03 ABC; 24 hrs after CV: 7320 ± 6.31 ABC; 48 hrs after CV: 7318 ± 10.91 ABC; 5 weeks after CV: 7296 ± 3.75 ABC; $P < 0.05$; Fig. 2B). Six patients were excluded from electrical CV because of left atrial thrombi detected by transesophageal echo. All atrial thrombi were located within the left atrial appendage. The clinical characteristics of these six patients were comparable to the remaining patients with AF (Table 2). In patients with atrial clot, levels of CD40 and CD40L and the inducible platelet activity after *ex vivo* stimulation were not different compared with the remaining patients in AF (data not shown).

Systemic Inflammatory Markers and Adhesion Molecules. Levels of hsCRP were significantly increased in AF compared with SR (AF: 5.44 ± 1.87 mg/l vs. SR: 1.62 ± 0.36 mg/l; $P < 0.05$), as were systemic VCAM levels (AF: 800.0 ± 21.1 ng/ml vs. SR: 679.1 ± 37.83 ng/ml; $P < 0.05$), ICAM levels (AF: 232 ± 12.6 ng/ml vs. SR: 196.4 ± 7.65 ng/ml; $P < 0.05$), and MCP-1 levels (AF: 239.99 ± 13.64 pg/ml vs. SR: 203 ± 9.29 pg/ml; $P < 0.05$).

Importantly, hsCRP, VCAM, ICAM, and MCP-1 levels were highest in the six patients with documented atrial thrombi (Fig. 3). Of note, MCP-1 (292.48 ± 23.24 pg/ml; $P < 0.05$) and hsCRP levels (15.13 ± 6.54 mg/l; $P < 0.001$) showed the most substantial differences compared with the remaining patients with AF. Successful CV caused a significant decline in hsCRP levels during follow-up (before CV: 5.44 ± 1.87 mg/l vs. 5 weeks: 2.08 ± 0.63 mg/l; $P < 0.05$). In contrast, ICAM, VCAM, and MCP-1 levels remained elevated after successful CV (VCAM before CV: 800.0 ± 29.13 ng/ml vs. 5 weeks: 779.68 ± 80.35 ng/ml, $P =$ ns; MCP-1 before CV: 239.99 ± 13.6 pg/ml vs. 5 weeks: 221.75 ± 24.46 pg/ml, $P =$ ns; ICAM before CV: 232 ± 12.6 ng/ml vs. 5 weeks: 204.08 ± 23.27 ng/ml, $P =$ ns; Fig. 4). However, a linear correlation between CD40 and ICAM, VCAM, or MCP-1 levels could not be demonstrated (data not shown). In contrast, VCAM levels correlated with patient age ($r = 0.49$; $P < 0.01$). Using univariate analysis, the presence of diabetes mellitus was related to MCP-1 ($P < 0.05$) and VCAM ($P = 0.059$). Smoking habits (pack years) correlated with baseline hsCRP levels ($r = 0.70$; $P < 0.01$). Importantly, VCAM and MCP-1 levels were independent predictors for the presence of atrial thrombi on multinominal regression analysis ($P < 0.05$).

Discussion

Main Findings. To the best of our knowledge, this is the first study showing increased platelet surface CD40 expression, as well as increased levels of adhesion molecules, in patients with persistent AF. Adhesion molecules, MCP-1, and hsCRP reached the highest levels in patients with overt atrial thrombi. Levels of hsCRP declined progressively after successful CV of persistent AF, whereas levels of adhesion molecules and MCP-1 remained elevated for at least 5 weeks. Importantly, VCAM and MCP-1 were independent predictors for atrial thrombi using

Table 2. Characteristics of AF Patients With and Without Clot[a]

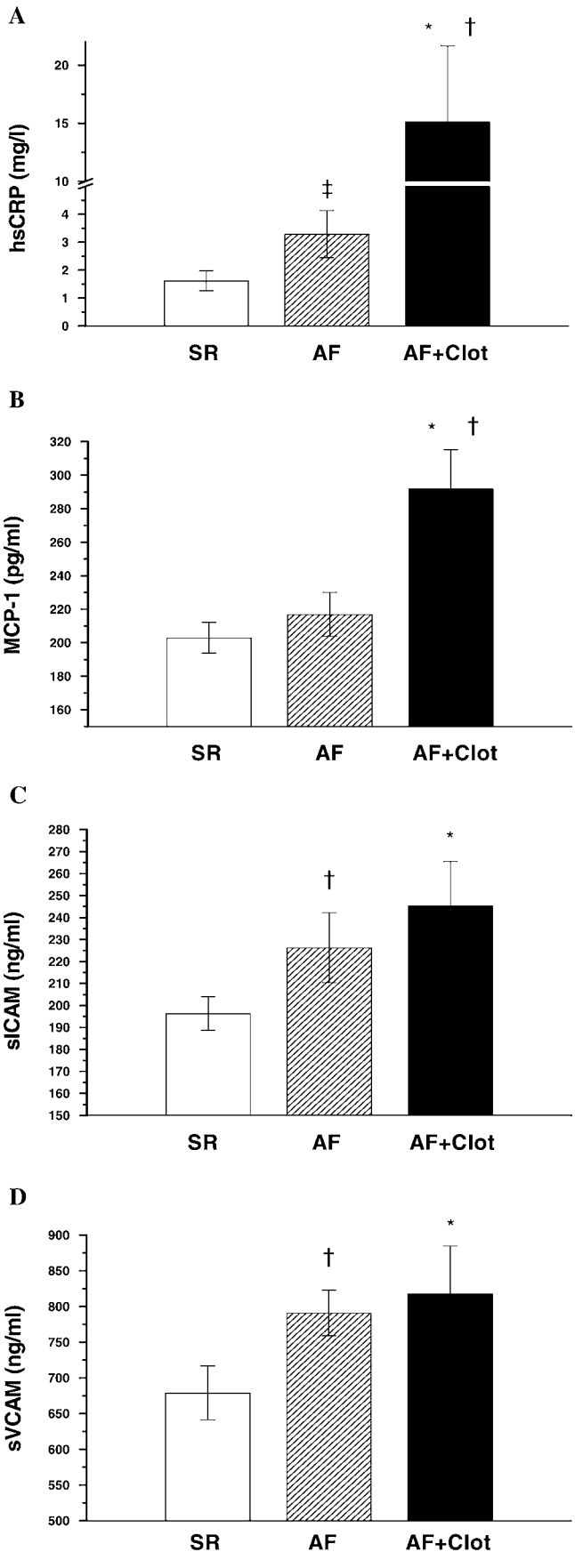
	AF and clot ($n = 6$)	AF ($n = 14$)	P
Age, years	66.83 ± 5.93	61.85 ± 2.05	—
Male/female, *n*	4/2	9/5	—
Weight, kg	84.83 ± 6.17	94.85 ± 3.99	—
BMI, kg/m^2	28.16 ± 1.77	31.35 ± 1.52	—
SBP, mm Hg	120.83 ± 9.52	139.07 ± 4.62	—
DBP, mm Hg	75.5 ± 4.99	84.85 ± 3.72	—
Current NYHA class	1.33 ± 0.55	1.38 ± 0.28	—
LAD(s), cm	4.76 ± 0.32	4.8 ± 0.17	—
LVEDD, cm	5.69 ± 0.65	4.61 ± 0.27	—
Diabetes, *n* (%)	3 (50)	3 (21)	—
Hypertension, *n* (%)	5 (83)	12 (86)	—
Left ventricular hypertrophy, *n* (%)	2 (33)	9 (45)	—
History of CAD, *n* (%)	2 (33)	1 (7)	—
Previous myocardial infarction, *n* (%)	1 (16)	0 (0)	—
Previous CABG/PTCA, *n* (%)	0 (0)	1 (7)	—
Valvular heart disease, *n* (%)	0 (0)	2 (14)	—
Previous thromboembolism/stroke, *n* (%)	2 (33)	0 (0)	—
History of smoking, *n* (%)	2 (33)	7 (50)	—
Hyperlipoproteinemia, *n* (%)	5 (83)	9 (45)	—
Class I antiarrhythmic drugs, *n* (%)	0 (0)	2 (14)	—
Class II antiarrhythmic drugs, *n* (%)	5 (83)	11 (78)	—
Class III antiarrhythmic drugs, *n* (%)	2 (33)	4 (29)	—
Class IV antiarrhythmic drugs, *n* (%)	0 (0)	1 (7)	—
ASA, *n* (%)	1 (16)	3 (21)	—
Coumadin, *n* (%)	4 (66)	10 (71)	—
ACE inhibitors/AT1 antagonists, *n* (%)	6 (100)	10 (71)	—
Dihydropyridine calcium antagonists, *n* (%)	1 (16)	1 (7)	—
Statins, *n* (%)	2 (33)	2 (14)	—

[a] Values are given as mean ± SEM or *n* (%). BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; NYHA, New York Heart Association; LAD, left atrial diameter; LVEDD, left ventricular end-diastolic diameter; CAD, coronary artery disease; CABG, coronary artery bypass graft; PTCA, percutaneous transluminal coronary angioplasty; ASA, acetyl salicylic acid; ACE, angiotensin-converting enzyme; AT1, angiotensin-1 receptor. None of the comparisions reached statistical significance.

multivariate analysis. Thus, in addition to hsCRP, MCP-1 and VCAM appear as new biomarkers, which may help to further identify patients with an increased risk for thromboembolic events.

Platelets, Biomarkers, and AF. CD40 and its ligand have been described as expressed on endothelial cells, smooth muscle cells, and fibroblasts, where they play a pivotal role in inflammatory responses, such as the expression of adhesion molecules, cytokines, matrix-degrading enzymes, and apoptotic mediators (21–23). It was not until recently that CD40 was found to be constitutively present on platelets, whereas preformed CD40L might be expressed seconds to minutes after platelet activation by diverse stimuli (12, 24). Once expressed, CD40L can interact with membrane-bound CD40 on endothelial cells, triggering an inflammatory reaction leading to local or systemic release of ICAM, VCAM, and MCP-1 (10, 12, 25). Upon platelet activation and thrombus formation, CD40/CD40L interaction among platelets may lead to shedding of CD40L, producing its soluble form, sCD40L. Whether this soluble form of CD40L is capable of inducing an inflammatory reaction acting as a *bona fide* cytokine or is inactivated upon cleavage is still a matter of discussion (24–28). It is also not finally clarified whether platelet–platelet interaction of CD40 and CD40L abrogates thrombus formation and inflammation by cleavage of CD40L, or whether further thrombus activation is achieved due to prothrombogenic effects of CD40 ligation with sCD40L (24, 28).

To the best of our knowledge, the present study is the first to analyze platelet surface expression of CD40 and CD40L in patients with AF. Our study shows that the CD40 system is activated during AF. However, the pattern of activation appears different compared with findings in patients with coronary artery disease, in whom CD40 and CD40L levels are increased (13–17). During AF, platelet CD40/CD40L appears very sensitive to TRAP but not to ADP, which may be clinically relevant for platelet inhibitory therapy with ADP receptor antagonists in patients with AF (29). Nevertheless, the absolute changes in the platelet CD40/CD40L system during AF were modest compared with the massive changes in platelet P-selectin expression after TRAP stimulation. Thus, the overall impact of the CD40 activation in AF might be limited, since studies in patients with acute coronary syndromes have shown an increase in platelet CD40/CD40L expression of up to 50%.



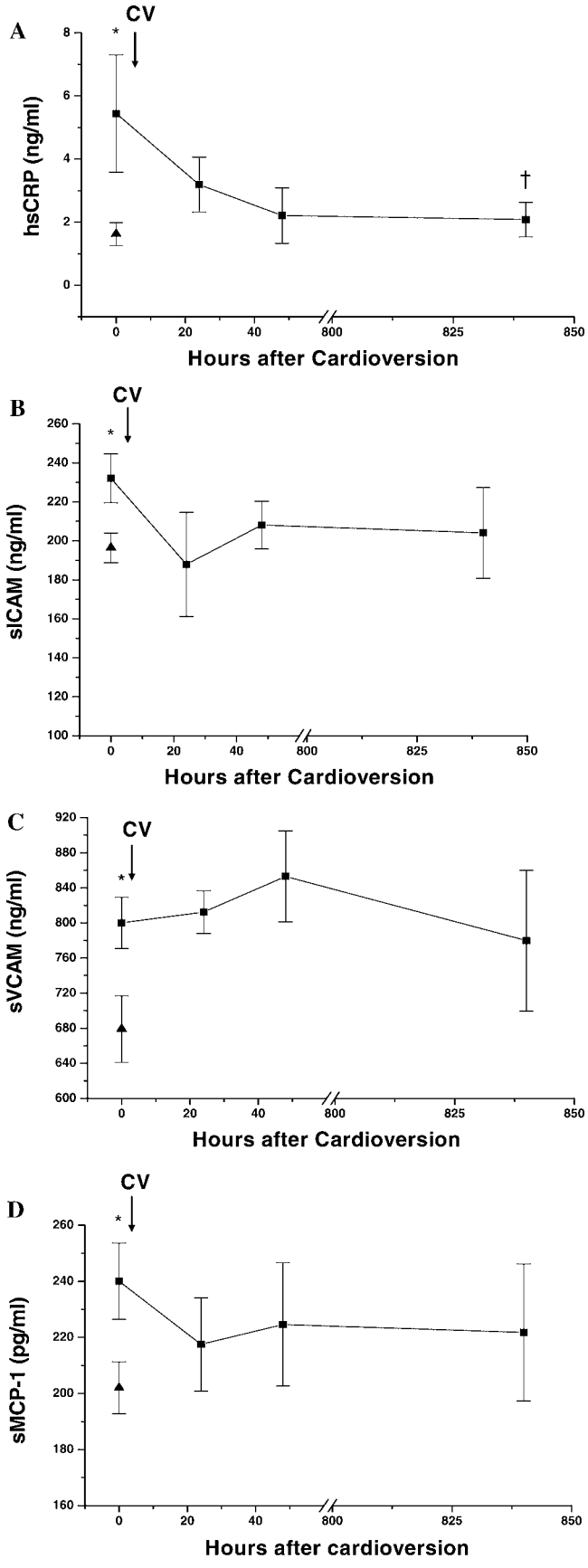
Of note, successful CV did not lower CD40 levels. Thus, CD40-dependent platelet–platelet interactions, as well as platelet–endothelium interactions remain activated for at least 5 weeks after cardioversion. This suggests that activation of this system does not solely depend on the arrhythmia. CD40 activation might also be maintained by long-lasting procoagulative alterations at the atrial endocardium.

In addition to CD40-dependent effects, platelet–leukocyte/monocyte interactions (characterized by activation of P-selectin and MCP-1) were enhanced during AF. The finding that MCP-1 levels are independent predictors for atrial thrombi is of particular interest (10). Nitric oxide (NO) is an important regulator for MCP-1 expression, and it has been shown that reduced availability of NO induces a substantial upregulation of MCP-1 expression (30). Thus, the progressive reduction in NO availability, which has been demonstrated to occur in fibrillating atrial tissue, might be one factor causing the increase of MCP-1 as well as adhesion molecules (30–33). Kamiyama showed that 8 hrs of rapid atrial pacing increases ICAM expression at the left atrial endocardium in rabbits (34). Interestingly, upregulation of ICAM was associated with leukocyte adherence to the atrial endocardium, which might be influenced by platelet P-selectin expression and MCP-1.

Nevertheless, AF is not an absolute prerequisite for the development of such prothrombogenic alterations at the atrial endocardium, because it was demonstrated that pressure overload ("stretch") *per se* causes increased expression of various endocardial proteins (35). Therefore, in many patients with AF it has to be considered that the development of the arrhythmia might be a secondary phenomenon related to preexisting structural and thereby prothrombogenic alterations of the atrial myocardium and endocardium ("endocardial remodeling"). Thus, in some patients a prothrombogenic milieu persists or is imminent, whereas the presence of AF on the surface electrocardiogram may serve only as a marker for such alterations (Fig. 5). This may help to explain the present finding that VCAM and MCP-1 levels remained elevated for weeks after successful CV of AF, because the underlying structural

←

Figure 3. (A) Levels of hs-CRP in patients with SR (white columns), AF patients (striped bars), and AF patients with atrial thrombus (black columns). Values are means ± SEM. *$P < 0.001$ AF with atrial thrombus vs. SR; †$P < 0.001$ AF with atrial thrombus vs. AF; ‡$P < 0.05$ AF without atrial thrombus vs. SR. (B) MCP-1 levels: SR (white columns), AF (stripped bars), and AF with atrial thrombus (black columns). Values are means ± SEM. *$P < 0.001$ AF with atrial thrombus vs. SR; †$P < 0.05$ AF with atrial thrombus vs. AF. (C) Serum ICAM (s-ICAM) levels: SR (white columns), AF (striped bars), and AF with atrial thrombus (black columns). Values are means ± SEM. *$P < 0.05$ AF with atrial thrombus vs. SR. †$P < 0.05$ AF without atrial thrombus vs. SR. (D) Serum VCAM (s-VCAM) levels: SR (white columns), AF (striped bars), and AF with atrial thrombus (black columns). Values are means ± SEM. *$P < 0.05$ AF with atrial thrombus vs. SR; †$P < 0.05$ AF without atrial thrombus vs. SR.



abnormalities of the atria are not instantaneously normalized by restoration of SR. Of note, MCP-1 and VCAM levels are independent predictors for atrial thrombi, which suggest a clinical importance for our findings. MCP-1 and VCAM appear as biomarkers, which may help identify patients with an increased risk for thromboembolic events. Further studies are warranted to assess the predictive value of these parameters in a larger cohort of patients with AF.

Previous studies have demonstrated increased systemic levels of inflammatory markers (interleukins, CRP, etc.) in patients with AF (18, 19, 33). However, the reversibility of such changes after cardioversion of AF has not been determined thus far. In the present study we can show that hsCRP levels decline after successful CV, resulting in dissociation between systemic hsCRP and adhesion molecules/MCP-1 levels (Fig. 5). Thus, we can hypothesize that to some extent, AF *per se* may influence/regulate hepatic CRP expression and, thereby, hsCRP may not directly relay on local atrial alterations. Furthermore, the presence of vascular thrombi can cause an increase in systemic CRP levels (36). This may help to explain why patients with atrial thrombi showed highest hsCRP levels. Nevertheless, hsCRP was not an independent predictor for atrial clot formation using multinominal regression analysis. Thus, in contrast to activation of platelets and increased adhesion molecules, elevated CRP might be a consequence rather than a cause of thrombus development. This is supported by previous analyses including more than 40,000 patients showing that CRP levels *per se* do not predict the development of vascular thrombi or thromboembolic events, although CRP levels increase in response to the occurrence of vascular thrombi (37, 38).

Limitations. The number of patients included in the study is limited, one reason being that fluorescence-activated cell sorting analyses of cell surface proteins are complex and therefore difficult to perform in a large series of patients. Analyses of large cohorts of patients usually are based on stable soluble blood markers (e.g., IL-6, CRP), whereas in our study cell surface markers, *ex vivo* stimulation techniques, and soluble serum markers were determined. It is known that coronary artery disease and other factors substantially influence systemic inflammatory markers. Thus, matched cohorts of patients with no or

←

Figure 4. (A) Serum hsCRP levels before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (squares); SR (triangles). Values are means ± SEM. *$P < 0.05$ AF before CV vs. baseline SR; †$P < 0.05$ AF before CV vs. AF after CV. (B) Serum ICAM levels before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (squares); SR (triangles). Values are means ± SEM. *$P < 0.05$ AF before CV vs. baseline SR. (C) Serum VCAM levels before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (squares); SR (triangles). Values are means ± SEM. *$P < 0.05$ AF before CV vs. baseline SR. (D) Serum MCP-1 levels before CV and 24 hrs, 48 hrs, and 5 weeks after successful electrical CV (squares); SR (triangles). Values are means ± SEM. *$P < 0.05$ AF before CV vs. baseline SR.

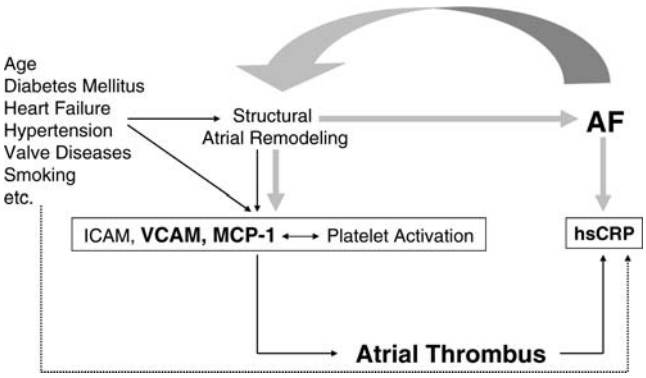


Figure 5. Hypothesis on causes and mechanisms of inflammation, AF, and clot formation. Preexisting comorbidities and risk factors lead to structural changes of the atrial tissue (structural remodeling) and, thereby, directly induce the expression of prothrombogenic markers (endocardial remodeling). In addition, structural alterations of atrial myocardium favor the occurrence of AF, which, in a positive feedback loop, enhances structural atrial remodeling. Recent data have already demonstrated that structural atrial changes are long lasting or may even be irreversible. This may encompass induced endocardial changes characterized by increased adhesion molecule (VCAM and ICAM) and MCP-1 expression as well as platelet activation, which appear as prerequisites for the development of atrial thrombi at the endocardium. However, hsCRP seems to be linked to the presence or absence of AF and atrial thrombi. Thus, in contrast to adhesion molecules and increased platelet activation, elevated CRP might be a consequence rather than a cause of thrombus development. This is supported by previous studies showing that CRP levels *per se* do not predict the development of vascular thrombi or thromboembolic events, although CRP levels increase after manifestation of vascular thrombi (37, 38).

minimal concomitant diseases were included only to determine the impact of AF on these systemic factors. Nevertheless, the limited number of patients does not allow one to draw definite conclusions regarding all analyzed parameters. For example, the pathophysiologic significance of the fall of CD40L after CV below the SR group remains unclear and should therefore be reproduced in larger cohorts of patients.

Angiotensin-converting enzyme inhibitors and AT1 antagonists were unequally present in the two groups, with an increased use in AF patients. Nevertheless, both drugs may lower ICAM and VCAM levels (39, 40). Therefore, the different distribution is less likely to explain the increased ICAM and VCAM levels in the patients with AF. Nevertheless, we cannot rule out that the use of these drugs in patients with AF might have contributed to a reduction of these factors and, thereby, potential differences to patients in SR might have been mitigated. In addition to angiotensin-converting enzyme inhibitors, coumadin therapy was used more often in patients with AF. The specific effect of coumadin on inflammatory markers and adhesion molecule expression has not been studied in detail. The data available so far are very limited and give conflicting results (41, 42). However, medical therapy was not changed throughout the study in any patient, and thus intraindividual effects induced by medication were excluded.

Conclusions. Prothrombogenic markers are substantially elevated in patients with AF, reaching highest levels in patients with AF and atrial thrombi. Interestingly, amounts of adhesion molecules and platelet CD40 levels remain elevated even 5 weeks after successful CV, which may imply a persistently increased risk for atrial thrombus formation. In addition to hsCRP, MCP-1 and VCAM may serve as new biomarkers, which may help to identify patients with an increased risk for thromboembolic events.

1. Brand FN, Abbott RD, Kannel WB, Wolf PA. Characteristics and prognosis of lone atrial fibrillation: 30-year follow-up in the Framingham Study. JAMA 254:3449–3453, 1985.
2. EAFT Study Group. Secondary prevention in non-rheumatic atrial fibrillation after transient ischemic attack or minor stroke. Lancet 342: 1255–1262, 1993.
3. Wyse DG, Waldo AL, DiMarco JP, Domanski MJ, Rosenberg Y, Schron EB, Kellen JC, Greene HL, Mickel MC, Dalquist JE, Corley SD. A comparison of rate control and rhythm control in patients with atrial fibrillation. N Engl J Med 347:1825–1833, 2002.
4. Lip GY, Lip PL, Zarifis J, Watson RD, Bareford D, Lowe GD, Beevers DG. Fibrin D-dimer and ß-thromboglobulin as markers of thrombogenesis and platelet activation in atrial fibrillation. Effects of introducing ultra-low dose warfarin and aspirin. Circulation 94:425–431, 1996.
5. Kumagai K, Fukunami M, Ohmori M, Kitabatake A, Kamada T, Hoki N. Increased intracardiovascular clotting in patients with chronic atrial fibrillation. J Am Coll Cardiol 16:377–380, 1990.http://circ.ahajournals.org/cgi/external_ref?access_num=2373815&link_type=MED
6. Sohara H, Amitani S, Kurose M, Miyahara K. Atrial fibrillation activates platelets and coagulation in a time-dependent manner: a study in patients with paroxysmal atrial fibrillation. J Am Coll Cardiol 29: 106–112, 1997.
7. Ikeda U, Yamamoto K, Shimada K. Biochemical markers of coagulation activation in mitral stenosis, atrial fibrillation and cardiomyopathy. Clin Cardiol 20:7–10, 1997.
8. Conway DS, Pearce LA, Chin BS, Hart RG, Lip GY. Plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 1321 patients with non-valvular atrial fibrillation: relationship to stroke risk factors. Circulation 106:1962–1967, 2002.
9. Goette A, Ittenson A, Hoffmanns P, Reek S, Hartung W, Klein H, Ansorge S, Geller JC. Increased expression of P-selectin in patients with chronic atrial fibrillation. Pacing Clin Electrophysiol 23:1872–1875, 2000.
10. Gawaz M, Neumann FJ, Dickfeld T, Koch W, Laugwitz KL, Adelsberger H, Langenbrink K, Page S, Neumeier D, Schömig A, Brand K. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 98:1164–1171, 1998.
11. McGregor L, Martin J, McGregor J. Platelet–leukocyte aggregates and derived microparticles in inflammation, vascular remodelling and thrombosis. Front Biosci 11:830–837, 2006.
12. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczek RA. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 391:591–594, 1998.
13. Mach F, Schönbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. Nature 394: 200–203, 1998.
14. Aukrust P, Müller F, Ueland T, Berget T, Aaser E, Brunsvig A, Solum NO, Forfang K, Frøland SS, Gullestad L. Enhanced levels of soluble

and membrane-bound CD40 ligand in patients with unstable angina possible reflection of T-lymphocyte and platelet involvement in the pathogenesis of acute coronary syndromes. Circulation 100:614–620, 1999.

15. Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, Simoons ML, for the CAPTURE Study Investigators. Soluble CD40 ligand in acute coronary syndromes. N Engl J Med 348: 1104–1111, 2003.
16. André P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. Circulation 106:896–899, 2002.
17. Schönbeck U, Libby P. CD40 signaling and plaque instability. Circ Res 89:1092–1103, 2001.
18. Conway DSG, Buggins P, Hughes E, Lip GYH. Relationship of interleukin-6 and C-reactive protein to the prothrombotic state in chronic atrial fibrillation. J Am Coll Cardiol 43:2075–2082, 2004.
19. Chung MK, Martin DO, Sprecher D, Wazni O, Kanderian A, Carnes CA, Bauer JA, Tchou PJ, Niebauer MJ, Natale A, Van Wagoner DR. C-reactive protein elevation in patients with atrial arrhythmias: inflammatory mechanisms and persistence of atrial fibrillation. Circulation 104:2886–2891, 2001.
20. Nygren H, Broberg M. Specific activation of platelets by surface-adsorbed plasma proteins. J Biomater Sci Polymer Edn 9:817–831, 1998.
21. Schoenbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci 58:4–43, 2001.
22. van Kooten C, Banchereau J. CD40-CD40 ligand. Leukoc Biol 67:2–17, 2000.
23. Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40–CD40 ligand signaling in atherosclerosis. Proc Natl Acad Sci U S A 94:1931–1936, 1997.
24. Henn V, Steinbach S, Büchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. Blood 98: 1047–1054, 2001.
25. Hollenbaugh D, Mischel-Petty N, Edwards CP, Simon JC, Denfeld RW, Kiener PA, Aruffo A. Expression of functional CD40 by vascular endothelial cells. J Exp Med 182:32–40, 1995.
26. André P, Prasad KSS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD. CD40L stabilizes arterial thrombi by a β3 integrin–dependent mechanism. Nat Med 8:247–252, 2002.
27. Prasad KSS, André P, He M, Bao M, Manganello J, Phillips DR. Soluble CD40 ligand induces β3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signalling. Proc Natl Acad Sci U S A 100:12367–12371, 2003.
28. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circ Res 92:1041–1048, 2003.
29. Kamath S, Blann AD, Chin BS, Lip GY. A prospective randomized trial of aspirin-clopidogrel combination therapy and dose-adjusted warfarin on indices of thrombogenesis and platelet activation in atrial fibrillation. J Am Coll Cardiol 40:484–490, 2002.
30. Koyanagi M, Egashira K, Kitamoto S, Ni W, Shimokawa H, Takeya M, Yoshimura T, Takeshita A. Role of monocyte chemoattractant protein-1 in cardiovascular remodeling induced by chronic blockade of nitric oxide synthesis. Circulation 102:2243–2248, 2000.
31. Cai H, Li Z, Goette A, Mera F, Honeycutt C, Feterik K, Wilcox JN, Dudley SC, Harrison DG, Langberg JJ. Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation: potential mechanisms for atrial thrombosis and stroke. Circulation 106:2854–2858, 2002.
32. Minamino T, Kitakaze M, Sato H, Asanuma H, Funaya H, Koretsune Y, Hori M. Plasma levels of nitrite/nitrate and platelet cGMP levels are decreased in patients with atrial fibrillation. Arterioscler Thromb Vasc Biol 17:3191–3195, 1997.
33. Roldan V, Marin F, Blanna AD, Garcia A, Marco P, Sogorb F, Lip GYH. Interleukin-6, endothelial activation and thrombogenesis in chronic atrial fibrillation. Eur Heart J 24:1373–1380, 2003.
34. Kamiyama N. Expression of cell adhesion molecules and the appearance of adherent leukocytes on the left atrial endothelium with atrial fibrillation. Jpn Circ J 62:837–843, 1998.
35. Fukuchi M, Watanabe J, Kumagai K, Katori Y, Baba S, Fukuda K, Yagi T, Iguchi A, Yokoyama H, Miura M, Kagaya Y, Sato S, Tabayashi K, Shirato K. Increased von Willebrand factor in the endocardium as a local predisposing factor for thrombogenesis in overloaded human atrial appendage. J Am Coll Cardiol 37:1436–1442, 2001.
36. Bucek RA, Reiter M, Quehenberger P, Minar E. C-reactive protein in the diagnosis of deep vein thrombosis. Br J Haematol 119:385–389, 2002.
37. Fox EA, Kahn SR. The relationship between inflammation and venous thrombosis. A systematic review of clinical studies. Thromb Haemost 94:362–365, 2005.
38. Vormittag R, Vukovich T, Schonauer V, Lehr S, Minar E, Bialonczyk C, Hirschl M, Pabinger I. Basal high-sensitivity-C-reactive protein levels in patients with spontaneous venous thromboembolism. Thromb Haemost 93:488–493, 2005.
39. daCunha V, Tham DM, Martin-McNulty B, Deng G, Ho JJ, Wilson DW, Rutledge JC, Vergona R, Sullivan ME, Wang YX. Enalapril attenuates angiotensin II-induced atherosclerosis and vascular inflmmation. Atherosclerosis 178:9–17, 2005.
40. Ferrario CM, Strawn WB. Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease. J Am Coll Cardiol 98:121–128, 2006.
41. Liem LK, Choong LH, Woo KT. Action of dipyridamole and warfarin on growth of human endothelial cells cultured in serum-free media. Clin Biochem 34:141–147, 2001.
42. Kumar S, Singh BK, Kalra N, Kumar V, Kumar A, Prasad AK, Raj HG, Parmar VS, Ghosh B. Novel thiocoumarins as inhibitors of TNF-alpha induced ICAM-1 expression on human umbilical vein endothelial cells (HUVECs) and microsomal lipid peroxidation. Bioorg Med Chem 13:1605–1613, 2005.