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Factor XIa and tissue factor activity in patients with coronary artery disease

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Summary

It has been established that inflammation and enhanced procoagulant activity are associated with the pathogenesis of atherosclerotic vascular disease. We evaluated and compared the contributions of the factor (F)XIa and tissue factor (TF) activity in plasma of patients with coronary artery disease (CAD). Citrate plasma was obtained prior to therapy from 53 patients with stable angina (29 with a history of previous myocardial infarction; CAD-MI) and 30 with acute coronary syndrome (ACS) within 12 hours from pain onset. Four ACS patients treated with heparin were excluded. FXIa and TF activity were determined in clotting assays based upon the prolongation of clotting time by inhibitory monoclonal antibodies. Twenty-five of 26 ACS patients (96%) and 22 of 29 CAD-MI patients (76%) had quantifiable FXIa (50 ± 33 and 42 ± 45pM, respectively). Ten of 26 (38%) ACS patients and only three of 53 (6%) stable CAD patients showed TF activity (<0.4pM). No FXIa or TF activity was observed in age-matched healthy controls (n=12). For both CAD-MI and ACS patients, there were correlations ($p<0.05$) between FXIa and interleukin-6 (R^2= 0.59 and 0.39, respectively) and between FXIa and TAT (R^2= 0.64 and 0.63, respectively). In conclusion, the majority of ACS and CAD-MI patients have circulating FXIa that correlates with markers of coagulation and inflammation.

Keywords

Coronary artery disease, factor XIa, tissue factor, inflammation markers, coagulation markers

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Introduction

Inflammatory processes and enhanced procoagulant activity are closely related to the development of atherosclerotic plaques. Plaque disruption and subsequent thrombosis are the leading cause of acute coronary syndromes (ACS), including unstable angina, acute myocardial infarction (MI) and sudden death (1). Pro-inflammatory cytokines cause a disruption of normal function of the arterial endothelium leading to the up-regulation of adhesion molecules, which contribute to plaque growth (2). Circulating levels of pro-inflammatory cytokines are increased in ACS patients and are predictors of the onset and outcome of coronary artery disease (CAD) (3, 4).

One of the functions of cytokines is stimulation of tissue factor (TF) expression (5), which is a potent initiator of the coagulation cascade and plays a major role in plaque thrombogenicity (6). As a consequence of elevated procoagulant activity, higher levels of coagulation markers such as prothrombin fragment 1.2 (F1.2), thrombin-antithrombin complex (TAT), fibrinopeptide A, the D-dimer, soluble fibrinogen, etc. are observed (7–10). Although there are studies suggesting elevated concentrations of soluble TF in plasma of CAD patients, the prognostic value of TF assays remains controversial (11–13). For example, Campo et al. suggested that plasma TF level is a predictor of mortality in patients with acute MI (13), while other authors report no significant differences in TF activity (12) or antigen (11) between ACS patients and stable controls.

It has been also suggested that thrombin-driven and contact pathway-independent activation of factor (F) XI can play a role in the onset of the ACS (14). Minnema et al. (14) found that 24% of patients with acute MI and 8% with unstable angina pectoris had evidence of FXIa presentation in their plasma. Their assay was based upon the immunochemical detection of FXIa in complex with C1 inhibitor; one of the numerous serine protease inhibitors present in plasma (15), suggesting that potentially only a fraction of FXIa present in plasma was detected by this pro-

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cedure. The evaluation of FXIa in plasma is further complicated by controversies related to the efficiency of various plasma protease inhibitors towards FXIa (15, 16). Wuillemin et al. suggest that 47% of FXIa added to plasma forms a complex with C1 inhibitor (16), whereas Scott et al. report that only 8% of FXIa is involved in the complex formation with this inhibitor (15).

In the present study, we determined FXIa and TF activity in contact pathway-inhibited plasma from stable CAD patients and those with ACS. Assay specificity was based upon the prolongation of clotting time by the addition of inhibitory antibodies to FXIa and TF, respectively. Correlations between FXIa and levels of both interleukin-6 and TAT (a thrombin generation marker) were established.

Methods

Subjects

All participants of the study gave informed consent, and the protocol was approved by the Ethics Committee of the Jagiellonian University. We studied 30 patients with ACS admitted to the Department of Hemodynamics of the University and 53 patients with angiographically confirmed stable CAD (>50% stenosis in at least one major coronary artery) recruited from an outpatient clinic. Twenty-nine of the stable CAD patients (55%) had a history of previous MI 0.5–3 years before enrollment. ACS patients had experienced chest pain for up to 12 hours prior to seeking treatment. Upon arrival at the hospital, patients were enrolled, and blood was taken before treatment was initiated. All patients took 300 mg aspirin before the study. None of the subjects received thienopyridines or other anticoagulants prior to blood collection. Four ACS patients received 5,000 U of unfractionated heparin prior to the blood draw and were excluded. Inclusion criteria for ACS patients were typical chest pain and either ST-segment elevation ≥0.1 mV or ST-segment depression ≥0.1 mV in at least two contiguous leads, and elevated cardiac troponin levels. ST-segment elevated MI (STEMI) was diagnosed in 14 patients, and the remainder were considered non-STEMI (NSTEMI). Exclusion criteria for all individuals were as follows: cardiogenic shock or heart failure (NYHA III/IV), any acute illness, cancer, hepatic or renal dysfunction, history of venous thromboembolism or stroke, oral anticoagulant administration, previous coronary artery bypass surgery. Stable angina patients (Canadian Cardiovascular Society classes II or III) were matched to the ACS patients for age and sex. None of these patients developed ACS or underwent angioplasty within the six months prior to the study. Twelve healthy controls were matched to the CAD patients for age and sex. The characteristics of both patient groups and those of healthy controls are shown in Table 1.

Laboratory methods

Blood was drawn from an antecubital vein with minimal stasis within 15 minutes (min) upon admission in the case of ACS patients and after an overnight fast between 7 to 9 a.m. in the case of stable CAD patients and healthy volunteers. 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 morphology, glucose, creatinine, albumin, aminotransferases, and creatine kinase were assayed by routine laboratory techniques. Fibrinogen was determined using the Clauss method. High-sensitivity C-reactive protein (CRP) was measured by latex nephelometry (Dade Behring, Marburg, Germany). Commercially available immunoenzymatic assays were used to determine plasma interleukin-6 (IL-6; R & D Systems, Abingdon, UK), F1.2 and TAT (Dade Behring) by investigators blind to all subject data. All the intra-assay and inter-assay coefficients of variation were below 7%.

Plasma clotting assays

Plasma was thawed at 37°C in the presence of corn trypsin inhibitor (CTI; prepared as previously described [17]; prevents contact pathway initiation of coagulation). $CaCl_2$ 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 (18). In parallel, inhibitory monoclonal anti-FXI (αFXI-2) 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 the $CaCl_2$ addition. αFXI-2 is specific for FXI/XIa and inhibits FIX activation by FXIa (19). αTF-5 binds specifically to TF and interferes with the TF/FVIIa complex formation (20). Clotting times were determined using the ST8 instrument (Diagnostica Stago, Parsippany, NJ, USA). FXIa and TF activity in plasma was calculated from calibration curves developed with human FXIa (a gift from Dr. R. Jenny from Haematologic Technologies, Inc., Essex Junction, VT, USA) or relipidated (17) TF_{1-242} (a gift from Dr. R. Lundblad from Baxter Healthcare Corp., Duarte, CA, USA) in pooled 10-donor normal plasma.

Fluorogenic assay

Forty µl of citrate plasma (10-donor normal or CAD patient) was added to 1,940 µl of HBS/$CaCl_2$ buffer (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$ and 0.1% PEG 6000; all reagents purchased from Sigma) and placed into a cuvette. When desired, purified human FXIa was added to plasma. Twenty µl of a fluorogenic substrate (6,1-D-LPR-propylaminonaphthalenesulfonamide; synthesized in house) was added at a final 100 µM concentration and the rate of substrate hydrolysis was evaluated in a spectrofluorometer FluoroMax-2 (Jobin Yvon-Spex Instruments S.A., Inc., Edison, NJ, USA) at λ_{ex} 350 nm and λ_{em} 470 nm with a 450 nm cut-off filter in the emission light beam. FXIa concentration was determined from a calibration curve built by sequential dilutions of purified human FXIa in 10-donor normal plasma.

Statistical analysis

The data are presented as mean ± standard deviation (SD), median (minimum-maximum), or percent as noted. Differences between continuous variable groups were examined using the Student *t*-test (two-tailed). Logarithmic transformations were performed on data that were distributed non-normally. Odds ratios were constructed for categorical variables.

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Table 1: Clinical parameters for individuals included in the study.

	Healthy (n=12)	ACS (n=26)	CAD (with prev. MI) (n=29)	CAD (no prev. MI) (n=24)
Age, years	60 (52–73)	59 (46–76)	64 (50–76)	60 (47–75)
Sex, M/F	9/3	21/5	21/8	14/10
BMI, kg/m^2	26.2 (22.8–32.3)	26.5 (21.4–29.8)	26.4 (22.1–29.8)	26.5 (21.8–30.6)
Current smokers, n(%)	4 (33%)	9 (35%)	11 (38%)	5 (21%)
Hypertension, n(%)	0 (0%)	19 (73%)	15 (52%)	14 (58%)
Diabetes, n(%)	0 (0%)	3 (12%)	3 (10%)	3 (12%)
Previous MI, n(%)	0 (0%)	4 (15%)	29 (100%)	0 (0%)
POAD, n(%)	0 (0%)	0 (0%)	4 (14%)	2 (8%)
Previous revascularization, n(%)	0 (0%)	3 (11%)	20 (69%)	9 (38%)
Total cholesterol, mM	5.41 (4.64–6.76)	6.20 (3.55–7.72)	4.41 (3.28–7.81)	5.1 (2.36–6.99)
LDL cholesterol, mM	3.51 (2.41–4.26)	3.86 (2.22–5.36)	2.53 (1.79–5.91)	2.95 (1.33–4.49)
HDL cholesterol, mM	1.405 (0.90–2.06)	1.14 (0.67–2.12)	1.17 (0.65–1.91)	1.26 (0.57–3.99)
Triglycerides, mM	1.425 (0.72–2.39)	2.22 (0.49–6.45)	1.56 (0.7–3.01)	1.36 (0.42–4.47)
Glucose, mM	5.15 (4.6–6.2)	6.65 (5.0–16.4)*	4.9 (3.8–10.5)	5.55 (4.0–7.7)
Creatinine, µM	64.5 (45–93)	76 (47–128)	78 (44–119)	83.5 (57–128)*
Troponin T, ng/l	0 (0–0)	4.52 (0.01–166.7)*	0 (0–0)	0 (0–0)
Aspirin, n(%)	0 (0%)	26 (100%)	26 (90%)	23 (96%)
Statins, n(%)	0 (0%)	6 (23%)	25 (86%)	22 (92%)
Beta-blockers, n(%)	0 (0%)	11 (42%)	20 (69%)	21 (92%)
ACE inhibitors, n(%)	0 (0%)	9 (35%)	19 (66%)	15 (88%)
Calcium antagonists, n(%)	0 (0%)	3 (12%)	4 (14%)	6 (25%)
Diuretics, n(%)	0 (0%)	3 (12%)	6 (21%)	6 (25%)
APTT, sec	30.8 (27.8–34.2)	29.1 (21.0–49.0)	33.5 (27.6–40.6)	32.4 (25.6–40.5)
Platelets, 10^3/µl	263 (189–310)	240 (131–341)	264 (189–403)	242 (83–413)
Fibrinogen, g/l	2.82 (2.14–3.36)	3.72 (2.57–5.85)*	3.81 (1.96–5.7)	3.20 (2.22–7.23)
C-reactive protein, mg/l	1.72 (0.88–2.31)	3.035 (0.5–34.81)*	1.9 (0.5–9.75)	2.01 (0.42–6.12)

*p<0.05 when compared to healthy.

Results

Baseline characteristics

Seventy-nine CAD patients were divided into three groups, i.e. those with ACS (n=26), stable CAD patients with a history of previous MI (n=29; CAD-MI) and stable CAD patients without previous MI (n=24; CAD-w/oMI). A group of healthy individuals (n=12) was also analyzed (Tables 1 and 2). Individuals in all four groups were balanced in terms of age and BMI. The percentage of current smokers was lower (21%) in CAD-w/oMI group than in other three groups (33–38%). No hypertension, diabetes or previous MI was diagnosed in healthy individuals. They also had the lowest concentration of fibrinogen (2.8 g/l). The ACS group had the highest frequency (73%) of hypertension among all three patient groups. The ACS group also had the highest total cholesterol (6.2 mM), LDL (3.9 mM), triglyceride (2.2 mM), glucose (6.65 mM) and CRP (3.0 mg/l) concentration and detectable troponin T amounts (4.5 mg/l). There were no significant differences in clinical parameters between the CAD-MI and the CAD-w/oMI groups with exception in number of patients with previous revascularization (69% and 38%, respectively). The APTT and platelet count were similar for all four groups.

Clotting activity

We observed that frozen citrate plasma from healthy individuals defrosted in the presence of 0.1 mg/ml CTI and 2 µM PCPS does not clot in 3,000 seconds (s) upon the addition of 15 mM $CaCl_2$. When plasmas from CAD patients were tested with the same stimulus, we observed that most plasma samples had clotting times shorter than 3,000 s with some as short as 300 s. These plasmas thus had elevated procoagulant activity when compared with plasma from healthy individuals. We hypothesized that this activity could be related to TF-bearing microparticles potentially present in the patient plasma. However, while addition of an inhibitory monoclonal anti-TF antibody prolonged the clotting time of some of these plasmas, the major-

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Table 2: Levels of IL-6, TAT, F1.2, FXIa and TF activity, and their correlations.

	ACS	Stable CAD		Healthy
		With previous MI	No previous MI	
N	26	29	24	12
FXIa (n)	25	22	5	0
FXIa (pM)	50.3 ± 32.8	41.7 ± 45.4	8.1 ± 19.9	0
TF act. (n)	10	2	1	0
TF act. (pM)	<0.4	<0.4	<0.4	0
IL-6 (pg/mL)	3.46 ± 1.19	1.83 ± 0.60	1.64 ± 0.76	1.25 ± 0.25
TAT (ng/mL)	6.75 ± 1.44	2.88 ± 0.58	2.76 ± 0.61	2.48 ± 0.19
F1.2 (nM)	1.40 ± 0.59	0.85 ± 0.33	0.73 ± 0.22	0.72 ± 0.13
R-Sq (XIa/IL-6)	0.385	0.594	ND	ND
R-Sq (XIa/TAT)	0.628	0.637	ND	ND
R-Sq (XIa/F1.2)	0.006	0.324	ND	ND

N, total number of individuals in each group; n, number of patients with FXIa or TF activity; ND, not determined.

ity of "active" plasmas were not affected by the addition of the anti-TF antibody. Moreover, even for those plasmas, which responded to the anti-TF antibody, the prolongation of the clotting time still did not extend to 3,000 s. This observation led to the conclusion that a TF-independent procoagulant activity was present in a significant fraction of CAD patients. Potential candidates for this activity were FIXa, FXa and FXIa and thrombin. Further experiments, which employed the corresponding inhibitory monoclonal antibodies, showed that all CAD patient plasmas with elevated procoagulant activity responded to the addition of an inhibitory anti-FXI antibody leading to the prolongation of the clotting time to >3,000 s for most of plasmas suggesting the presence of active FXIa in these samples.

The persistence of a serine protease in plasma, which contains an abundance of inhibitors for serine proteases, is an unusual observation and raises the question of whether FXIa can survive in that environment. Experiments were conducted to test the stability of FXIa in citrate plasma. Plasma from healthy donors to which exogenous FXIa was added showed that 80 to 90% of FXIa survives in citrate plasma over the time required for plasma preparation from blood (30–40 min). Additionally, the *de novo* formation of FXIa activity was not observed in citrate plasma either in the presence or absence of contact pathway inhibition at room temperature over a period of 75 min. These data indicate that the FXIa observed in plasma from ACS and stable CAD patients reflects the blood levels of this enzyme and is not an artifact of plasma preparation caused by the contact activation.

FXIa activity

Clotting activity

To quantitate FXIa concentration in CAD patient plasmas, a calibration curve was constructed by the addition of varying concentrations of purified plasma FXIa to 10-donor plasma from healthy individuals (FXI present at 94% of mean physiologic concentration). This plasma did not clot in 3,000 s with no FXIa added. The addition of 10 pM FXIa to this plasma lead to a clotting time of 2,200 s. With increasing FXIa concentration, the clotting time steadily decreased to 260 s at 500 pM FXIa. When 0.1 mg/ml αFXI-2 was added to plasma treated with 500 pM FXIa, no clot was observed over 3,000 s. The addition of 0.05–0.2 mg/ml αFXI-2 to 10-donor plasma initiated to clot with relipidated TF had no effect on the clotting time indicating that this antibody does not interfere with TF pathway to thrombin and is specific for FXIa. Repeated titrations of FXIa in 10-donor plasma gave reproducible clotting times (S.D.<10% of mean values).

The analyses of FXIa in patient and healthy volunteer plasma showed that 25 of 26 (96%) patients with ACS contained quantifiable (>10 pM) FXIa (Fig. 1A; Table 2). The concentration of FXIa varied from 16 to 120 pM with an average value of 50 ± 33 pM (mean ± SD). Analyses of plasma from 29 CAD-MI patients showed that 22 (76%) contained quantifiable amounts of FXIa at concentrations ranging from 15 to 190 pM with a mean value of 42 ± 45 pM. In contrast, only five of 24 CAD-w/oMI patients (21%) contained detectable amounts of FXIa. The odds ratio of detecting plasma FXIa in stable survivors of MI was 11.9 (95% confidence interval, 3.2–43.9). No FXIa activity was detected in plasma from 12 healthy volunteers or pooled 10 healthy donor plasma.

Amidolytic activity

To test our conclusion that the clotting activity observed in the plasmas of CAD patients and its response to the anti-FXI antibody is related to the presence of an active enzyme, we evaluated 10-donor and a CAD patient plasma for the amidolytic activity in a fluorogenic assay. No substrate hydrolysis was observed when 10-donor plasma was mixed with the substrate solution. An addition of 50 pM purified FXIa to this plasma lead to substrate hydrolysis at a rate of 79 pM/s. When CAD patient plasma was tested in this assay, the rate of substrate hydrolysis observed was 150 pM/s, suggesting that the concentration of active FXIa in this plasma is 95 pM. The clotting assay of this plasma indicated that FXIa concentration is 140 pM. An incubation of this CAD patient plasma for 30 min at 25°C lead to a decrease in FXIa concentration by 10% (to 85 pM). These data are in an agreement

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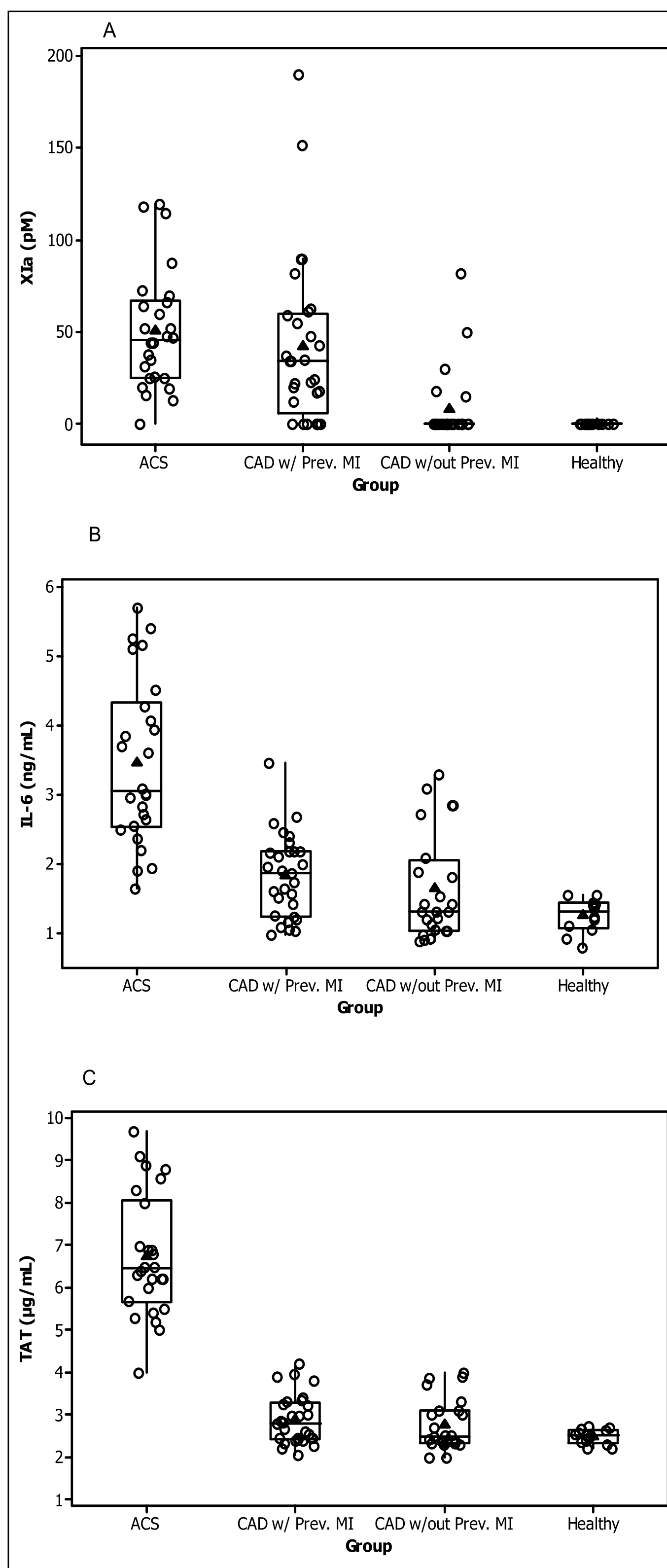

Figure 1: FXIa (A), IL-6 (B) and TAT (C) concentrations in ACS, CAD-MI and CAD-w/oMI patients and healthy individuals. Boxes encompass the data between the first and third quartiles with an additional line denoting the median. The vertical line extends to the highest and lowest data point within the limit (1.5 times the interquartile range from the first and third quartile). The triangles represent the mean values.

with the clotting experiment results both suggesting that in citrate plasma FXIa activity is only minimally compromised during the time required for plasma preparation (~30 min).

TF activity

The reliable quantitation limit of our TF activity assay based upon the titration of relipidated TF_{1-242} into CTI-inhibited 10-donor plasma is 0.4 pM. At this TF concentration, plasma clots in 1,420 s. The addition of 4 pM TF to 10-donor plasma decreases the clotting time to 215 s. In the presence of 0.1 mg/ml αTF-5, no clot is observed in 3,000 s when 4 pM TF is added. Repeated titrations of relipidated TF in 10-donor plasma gave reproducible clotting times (SD<10% of mean values).

Plasma from 10 of 26 ACS patients (38%) displayed detectable TF activity (Table 2). However, only four had TF activity at quantifiable concentrations; ranging from 0.5 to 0.9 pM. The concentrations of TF in the remaining samples were below the reliable quantitation limit of 0.4 pM (the lowest TF concentration, which can be quantitated reproducibly). For CAD-MI patients, two of 29 (6.9%) and for CAD-w/oMI patients one of 24 (4.2%) displayed TF activity below 0.4 pM. All three of these plasmas contained FXIa in the range from 48 to 82 pM. No TF activity was observed in the plasma from healthy individuals.

Markers of inflammation and coagulation and their correlation with FXIa

The concentration of IL-6 in plasma from patients with ACS (3.46 ± 1.19 pg/ml) was significantly higher ($p<0.001$) than any other group (Fig. 1B and Table 2). The IL-6 concentrations in CAD-MI and CAD-w/oMI groups were similar (1.83 and 1.64 pg/ml, respectively). The lowest IL-6 levels were observed in plasma from healthy individuals (1.25 ± 0.25 pg/ml). There were positive correlations ($p<0.05$) between the FXIa and IL-6 concentrations in both groups of patients, ACS (R^2=0.39; Fig. 2A) and CAD-MI (R^2=0.59; Fig. 2B).

Similar to observations for IL-6, the concentration of TAT (6.7± 1.4 ng/ml) in the ACS group was significantly higher ($p<0.001$) than in any other group (Fig. 1C). There was no significant difference between the TAT concentration in stable CAD patients and healthy volunteers (2.8 and 2.5 ng/ml, respectively). The correlation coefficient R^2 between FXIa and TAT ($p<0.05$) was 0.63 for the ACS group (Fig. 2C) and 0.64 for the CAD-MI group (Fig. 2D).

The pattern of the F1.2 concentration in the analyzed groups was similar to that of TAT, i.e. its concentration in the ACS group was significantly higher ($p<0.001$) than in other groups (Table 2). There was no significant correlation between FXIa and F1.2 in the ACS group. The R^2 for the CAD-MI group was 0.32 ($p<0.05$).

Discussion

Our data indicate that there are significant differences in the frequencies at which FXIa and TF are found in the plasma of stable CAD patients, those with ACS and healthy individuals. While no detectable FXIa or TF activity is observed in plasma from healthy volunteers, the majority of ACS patients display FXIa while a significant fraction display TF activity in plasma. A

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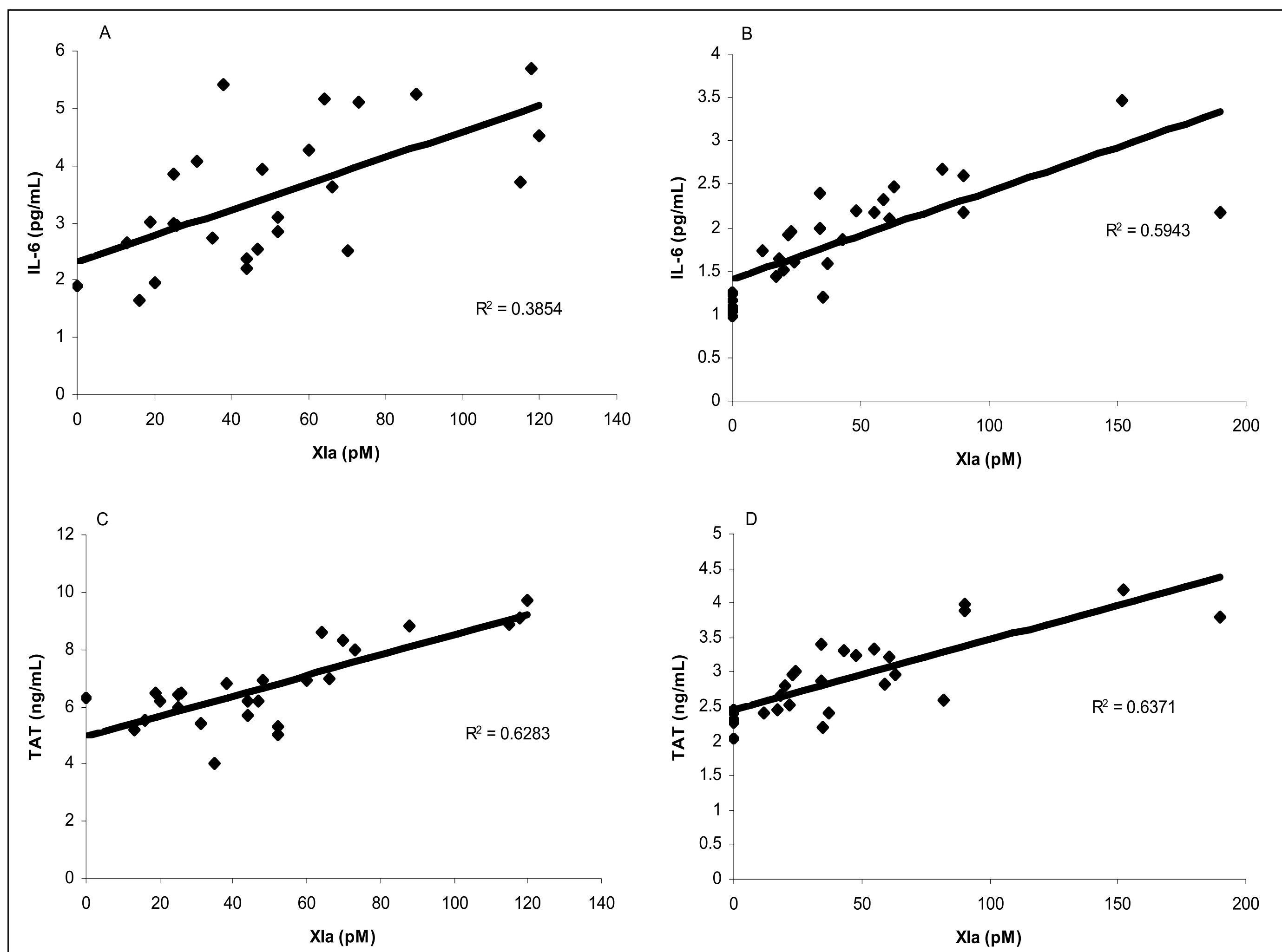

Figure 2: Correlation between FXIa and IL-6 (A and B) and between FXIa and TAT (C and D) in ACS (A and C) and CAD-MI (B and D) patients.

further stratification of stable CAD patients indicates that the majority of those with the history of previous MI have circulating FXIa, whereas only 21% of stable CAD patients without previous MI display FXIa activity.

The most surprising observation, which permitted this study to be conducted, is that an active serine protease, FXIa, is present in the plasma of CAD patients, i.e. an environment containing high concentrations of irreversible inhibitors for serine proteases (21). The pathway of FXIa generation in these patients is not clear.

In vitro, FXIa can be generated from its precursor FXI either by thrombin (22) or by FXIIa in conjunction with prekallikrein and high-molecular-weight kininogen, the initiating enzymatic complex of the contact pathway (23). While bleeding pathology does not seem to be associated with deficiency states of the initiating contact pathway proteins, it has been suggested that FXII deficiency is a risk factor for thrombosis, ACS and CAD (24–27). Recent studies using knock-out FXII- and FXI-deficient mice suggest that FXII plays a central role in pathologic thrombus formation, and that FXI activation by the contact pathway is a part of this process (28, 29). Studies reported by several groups attempting to establish correlations between FXIIa and coronary risk have led to contradictory results. Cooper et al. suggested that FXIIa is associated with increased coronary risk but found that low levels of FXIIa are not protective (30). A study published by Lowe et al. concluded that FXIIa is not associated with coronary risk (31). Similarly, it has been reported that ACS is not associated with contact pathway activation (32) and that the markers of contact pathway activation, including FXIIa, are not increased in ACS patients with elevated FXI activation (14).

The correlation between FXIa and IL-6 in our study together with previously published observations may imply that CAD patients with circulating FXIa have an increased TF expression on blood and vascular cells (6, 33). Additionally, the correlation between FXIa and TAT levels suggest a connection between thrombin generation and FXI activation. A possible explanation for FXIa activity in CAD patients is that with elevation of inflammatory cytokines, TF expression and, as a consequence, thrombin generation is enhanced, causing enhanced activation of FXI. This conclusion is supported by the data published by Cawthern et al.

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indicating that in contact pathway-inhibited whole blood initiated with TF, FXIa is generated primarily by thrombin (17) in contrast to the conclusion of Pedicord et al. based upon studies in citrate plasma that the contact pathway is the primary activator of FXI during haemostasis (34).

The absence of correlation between plasma FXIa and TF activity may be related to the properties and *in vivo* location of TF expression. TF is a membrane protein and may be expressed/exposed on the surface of perivascular and endothelial cells and monocytes upon cytokine stimulation (6, 33). Soluble TF, which lacks the ability to bind to cell or artificial membranes, has little (if any) activity in procoagulant processes (35, 36) It has been suggested by several groups of investigators that limited TF activity observed in plasma is associated with TF located on microparticles, and that microparticles bearing TF circulate *in vivo*, primarily in blood of patients with inflammation (37–39). Under conditions of a routine plasma preparation, microparticles are not removed with blood cells, remain in plasma preparations (37) and, as a consequence, transfer TF activity from blood to plasma. However, there is no evidence suggesting a correlation between TF concentration and activity in plasma and those in blood.

A study by Minnema et al. reported that only a small fraction of patients with acute MI and unstable angina pectoris contained detectable amounts of a FXI activation marker (FXIa-C1 inhibitor complex) (14). The discrepancy between the results of that study and our observations is most likely related to the technical approaches used to measure FXIa in plasma. Our assay measures the FXIa activity *per se*, whereas Minnema et al. quantitated the FXIa-C1 inhibitor complex. C1 inhibitor is one of several serine protease inhibitors that form covalent complexes with FXIa (15, 16). Although Wuillemin et al. suggest that C1 inhibitor is the most efficient in-vivo inhibitor of FXIa (16, 40), others report that only 8% of FXIa is present in complex with C1 inhibitor (15). Thus, the FXIa-C1 inhibitor complex may report only a fraction of FXIa present in plasma.

It is surprising that the abundance of inhibitors circulating *in vivo* allows FXIa to survive as an active enzyme. The absence of FXIa activity in plasma from healthy individuals indicates that FXIa detected in CAD patients is not an artifact of plasma preparation but circulates *in vivo*. Of course, our assay may only detect a part of FXIa generated. However, a comparison of FXIa concentrations in ACS patients of our study with the levels of the FXIa-C1 inhibitor complex reported for similar patients (14) suggests that the majority of FXIa formed circulates *in vivo* as a free enzyme or in reversible complex(es) with inhibitor(s). This conclusion is also supported by the data, which indicate that FXIa disappearance from plasma is a relatively slow process.

The data of the current study suggest that a FXIa assay might be used as a predictor of an elevated thrombotic risk and primarily applied to apparently stable CAD patients. In comparison with routine clinical laboratory tests, this FXIa assay is rapid, relatively simple and has the potential to be incorporated as a point-of-care assay (similar to that described by Holmes et al. [41]) for the stratification of CAD patients. However, larger studies are needed to assess the prognostic value of FXIa and TF activity in CAD patients. It would be also of interest to investigate whether FXIa activity is associated with poor clinical outcome in ACS.

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