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Procoagulant activity of circulating microparticles is associated with the presence of moderate calcified plaque burden detected by multislice computed tomography

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Abstract

Background   Circulating microparticles (MPs) have been reported to be associated with coronary artery disease (CAD). In this study, we explored the relationship between MPs procoagulant activity and characteristics of atherosclerotic plaque detected by 64-slice computed tomography angiography (CTA). **Methods**   In 127 consecutive patients with CAD but without acute coronary syndrome and who underwent 64-slice CTA, MPs procoagulant activity in plasma (by a thrombin generation test), soluble form of lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) and N(epsilon)-(carboxymethyl) lysine (CML) circulating levels (by ELISA) were measured. A quantitative volumetric analysis of the lumen and plaque burden of the vessel wall (soft and calcific components), for the three major coronary vessels, was performed. The patients were classified in three groups according to the presence of calcium volume: non-calcified plaque (NCP) group (calcium volume (%) = 0), moderate calcified plaque (MCP) group (0 < calcium volume (%) < 1), and calcified plaque (CP) group (calcium volume (%) ≥ 1). **Results**   MPs procoagulant activity and CML levels were higher in MCP group than in CP or NCP group ($P = 0.009$ and $P = 0.027$, respectively). MPs procoagulant activity was positively associated with CML ($r = 0.317$, $P < 0.0001$) and sLOX-1 levels ($r = 0.216$, $P = 0.0025$). **Conclusions**   MPs procoagulant activity was higher in the MCP patient group and correlated positively with sLOX-1 and CML levels, suggesting that it may characterize a state of blood vulnerability that may locally precipitate plaque instability and increase the risk of subsequent major cardiovascular events.

1   Introduction

Microparticles (MPs) are small membrane-derived vesicles (0.1–1 μm) shed by activated or apoptotic cells that express anionic phospholipids and an antigenic profile, characteristic of their cellular origin.[1] Circulating MPs are both a result of pathological modifications affecting the vascular compartment, and a pool of bioactive effectors able to modulate vascular homeostasis and contribute to cardiovascular disorders, promoting inflammation, thrombosis and vascular dysfunction themselves.[2] In pathological conditions, MPs are a source of procoagulant activity and are released both from vascular and peripheral blood cells and from atherosclerotic plaque after rupture.[3] Several studies show that both the number and procoagulant activity of MPs increase in patients with cardiovascular disease,[4] including stable and unstable coronary artery disease (CAD).[5] Circulating endothelial MPs have been associated with a higher risk for future adverse cardiac events in patients with stable CAD, becoming an independent predictor of acute coronary syndromes (ACS) and cardiovascular death.[6,7]

Multi-slice computed tomography angiography (CTA) has emerged as a promising non-invasive tool that produces high-resolution imaging of the coronary artery and wall and allows more reliable detection of both obstructive and non-obstructive subclinical CAD at an earlier stage than does invasive angiography.[8–10] CTA provides additional information about atherosclerotic plaque composition and permits the detection of both calcified plaque and non-calcified coronary atherosclerotic plaque, with good agreement with intravascular ultrasound (IVUS).[11]

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While it is well known that MPs released in the blood stream after plaque rupture contribute to thrombus formation in the ACS population,[12] little is known about the *in vivo* role of circulating MPs in the progression of atherosclerosis before an acute event has occurred. Several studies using *in vitro*-generated MPs of different cellular origins support the concept that MPs may modify the endothelial function and promote the recruitment of inflammatory cells in the vascular wall,[13,14] exacerbating the impaired endothelial function and favouring the progression of atherosclerotic.[15] To clarify this, we measured the procoagulant activity of MPs and explored their association with morphological characteristic plaque detected by CTA, in a cohort of subjects who presented chest pain syndrome and in whom ACS was excluded. Further, the relationship between MPs and inflammatory markers was explored.

2 Methods

2.1 Study population

We recruited 200 consecutive patients (age 63.3 ± 12 years; 63.5% men) with chest pain syndrome, proven or suspected CAD, who should have been screened by 64-slice CTA according to guidelines.[16]

The exclusion criteria were as follows: patients who either were suspected to have ACS or had severe symptoms before CTA ($n = 10$), patients with chronic total occlusion ($n = 13$), elevated pre-procedural cardiac biomarkers ($n = 14$), previous stenting ($n = 23$), and inestimable-quality CTA imaging ($n = 13$). Thus, the final study population comprised 127 patients. The institutional ethic committee approved the study protocol and all patients provided informed consent for the study.

2.2 Cardiovascular risk factors

We obtained information and clinical measures on risk factors and medication during the patient's initial visit. Hypertension was defined as systolic blood pressure over 140 mmHg and/or diastolic blood pressure over 90 mmHg and/or treatment with antihypertensive medication. Antihypertensive therapy included the following classes of drugs or any combination of them: ACE inhibitors, calcium-antagonists, beta-blockers, and diuretics. Platelet aggregation inhibitors, such as aspirin (< 500 mg/d), were recorded. Hypercholesterolemia was defined as fasting cholesterol over 200 mg/dL or use of cholesterol-lowering drugs (statins, fibrates, bile acid sequestrants and nicotinic acid derivatives). Diabetes was defined as fasting serum glucose levels over 126 mg/dL, or use of antidiabetic medi-

cation (insulin or any oral antidiabetic medication). Subjects were classified as smokers if they had smoked at least one cigarette per day in the year before the study.

Information was collected on previous cardiovascular and cerebrovascular events. Family history of CAD was defined as having a first-degree female (< 65 years) or male (< 55 years) relative with a documented history of myocardial infarction, sudden cardiac death and surgical or percutaneous coronary revascularization. The Framingham risk score was calculated for each subject using the risk score of Wilson, *et al*.[17]

2.3 Laboratory assays

Blood samples were drawn before CTA procedure and collected in tubes without additives, containing heparin or citrate (for routine biochemistry). Total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, and glucose were determined using routine laboratory procedures. Plasma and serum samples were centrifuged at 4°C, immediately divided in aliquots and stored at −80°C until analysis. For MPs analyses, a venous blood sample was obtained in citrated tubes, then centrifuged at 1,500 r/min at room temperature for 15 min. Plasma supernatant was then again rapidly centrifuged for 2 min at 13,000 r/min and plasma obtained was separated without disturbing the buffy coat and immediately frozen at −80°C until analysis. All laboratory determinations were performed in a blinded fashion.

2.4 MP activity assay

MP-associated procoagulant activity was assayed in plasma sample after thawing in a 37°C water bath for 15 min, with the Zymuphen MP-activity kit (Hyphen BioMed, Neuvillesur-Oise, France) according to the manufacturer's instructions. This method is based on a prothrombinase assay after MP capture from platelet-poor plasma (PPP) on a micro-titration plate coated with streptavidin and biotinylated annexin V. Next, a solution containing calcium and bovine FXa-FVa was added, followed by human prothrombin. Thrombin generation was detected by a chromogenic substrate. The results were expressed as nmol/L per phosphatidylserine equivalents.

2.5 Soluble form of lectin-like, oxidized, low-density lipoprotein receptor-1 (sLOX-1) assay

Serum sLOX-1 levels were measured by an in-house double-sandwich ELISA kit as described previously.[18] The inter-assay coefficient of variation value was 7.2%. The lower limit of detection for LOX-1 was 30 pg/mL.

2.6 *N* (epsilon)-(carboxymethyl) lysine (CML) assay

Plasma CML levels were measured, as previously described,[19] by a developed competitive ELISA using the mouse F(ab')2 anti-AGE monoclonal antibody 6D12 (ICN Biochemical Division, Aurora, Ohio, USA), which recognizes specifically CML-protein adducts. Intra-assay and inter-assay coefficients of variation were 3.2% and 8.7%, respectively. The lower limit of detection of CML was 0.5 µg/mL.

2.7 64-Slice CTA scan

The patients underwent 64-slice CTA (LightSpeed VCT 64, GE Healthcare, Milwaukee, WI, USA) with the following scan parameters: retrospective ECG gating; 912 channel detectors along the gantry and 64 channel detectors along the z-axis; tube voltage, 120 kV; tube current, 350–750 mA (depending on patient size); scan FOV, 50 cm; gantry rotation, 0.35 s/rotation; matrix, 512 × 512; slice thickness, 0.625 mm; range of helical pitch, 0.18–0.24. When appropriate the following premedications were administered: metoprolol, up to 5 mg intravenous, to lower the heart rate below 65 beats/min; isosorbide dinitrate, up to 1 mg intravenous to guarantee maximal epicardial vasodilatation. Non-ionic iodinated contrast medium (Iomeprol 400, Bracco, Italy) was injected via a peripheral vein according to a triphasic protocol using a programmable injector (Nemoto Dual Shot Injector, Nemoto Kyorindo Co. Ltd., Japan) with a two-way syringe system: in the first phase a rapid injection of contrast medium (from 5 to 8 mL/s) was performed; in the second phase 10 mL of contrast at 1 mL/s were injected simultaneously with 25 mL of saline at 2.5 mL/s; in the third phase 35 mL saline flush was administered at 4 mL/s (maximal total volume of contrast medium 110 mL). To time the scan, a region of interest was placed in the right ventricular cavity to detect peak enhancement. Scans were performed during breath hold; patients were monitored continuously through single-lead electrocardiography. The scan parameters were programmed in order to limit radiation exposure to 15 mSv on average. After the procedure, patients had an intravenous infusion of saline (500 mL) to improve hydration and prevent contrast-induced nephropathy. Moreover, all patients were instructed to repeat a measurement of serum creatinine between 2 and 7 days following the examination.

2.8 Image reconstruction and analysis

Trans-axial CT images were reconstructed using a slice thickness of 0.625-mm and 0.4-mm increments. The data were then transferred to a dedicated workstation (Advantage Workstation 4.3, GE Healthcare, Milwaukee, WI, USA) for post-processing. Lumen size (diameter and area) of the major coronary arteries was measured on "Multiplanar Reformatting Images" reconstruction using an automatic interactive program. A lumen reduction ≥ 50% was classified as a significant stenosis. In vessels showing multiple stenosis in series, only the most severe was considered. Volumetric analysis of the coronary vessel wall was evaluated using the Color Code Plaque analysis software (GE Healthcare, Milwaukee, WI, USA).[20] This densitometric method, based on the Hounsfield scale, allows the computation of a cylindrical volume around the vessel lumen and the relative contribution of calcium, fibro-fatty and fibromuscular components of the vessel wall. The algorithm of vessel wall analysis was tuned in each patient according to densitometric parameters measured by a region of interest (ROI) on the aortic root, left ventricle (LV) myocardium and epicardial fat. Using this system, CT density < 60 HU was attributed to the fibro-fatty component, between 60 and 200 HU to the fibro-muscular component while calcified plaques were differentiated from the lumen when they had a density > 800 HU. The program output gave a quantitative volumetric measurement (mm^3) of each component. In this paper, we considered the total plaque burden as the sum of the soft component (defined as fibro-fatty and fibrotic component) and calcified component in the three major coronary vessels. Each component was also expressed as percentage of vessel volume and utilized in per-patient analyses.

2.9 Statistical analysis

Data were analysed with the use of statistical software SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

The Kolmogorov–Smirnov test of normality was used to verify whether the distribution of variables followed a Gaussian pattern. Data with a normal distribution are given as mean ± SD. Variables with a skewed distribution are expressed as median and interquartile range. The χ^2 test was done to compare categorical data among the three groups. One way analysis of variance (ANOVA) was used to compare continuous data among the three groups. Fisher's multiple comparison was applied for those with significant findings in one-way ANOVA. Pearson's correlation analysis was used to test the relationship between clinical, laboratory, and CTA parameters. A two-tailed *P*-value < 0.05 was considered statistically significant.

3 Results

A total of 381 major coronary vessels were evaluated in 127 patients. Coronary calcification (calcium volume) was

tallied in cubic centimetre by CT analysis, and the patients were stratified into three groups by their calcium volume percentage (calcium volume/vessel volume × 100). The non-calcified plaque (NCP) group had a calcium volume (%) = 0; the calcified plaque (CP) group had a calcium volume (%) ≥ 1; and the moderate calcified plaque (MCP) group (0 < calcium volume (%) < 1). Subject characteristics within the three groups are shown in Table 1. Compared with those in the NCP group, patients in the MCP group were prevalently male and older. Moreover, they had higher prevalence of previous myocardial infarction and a lower prevalence of smoking.

As expected, the CP group showed prevalence of hypertension, stenosis ≥ 50% and tended to have a major Framingham risk score. There were no significant differences in basic metabolic panel among the three groups (Table 1). As expected, the percentage of lumen volume was lower in CP group than in NCP group ($P = 0.013$), whereas the plaque burden was higher ($P = 0.012$), (Table 2). The MPs procoagulant activity was higher in the MCP group than NCP or CP group ($P = 0.005$ and $P = 0.01$, respectively) (Table 3).

Similarly, the CML plasma levels were higher in the MCP group than NCP or CP groups ($P = 0.035$ and $P = 0.013$, respectively), (Table 3). Instead, there were no statistically significant differences in sLOX-1 levels among the three plaque groups. The correlation analysis, performed on all patients, revealed a direct association between MP procoagulant activity with both CML ($r = 0.317$, $P < 0.0001$) and sLOX-1 levels ($r = 0.216$, $P = 0.0025$) (Figure 1 A and B) but not with the other parameters indicated in Table 1 and 2.

4   Discussion

In this study, we evaluated the relationship between MP procoagulant activity and the characteristics of plaque composition detected by CTA in stable CAD patients. We found that MP procoagulant activity is higher in the patient group with moderate calcified plaque. Although previous studies have shown an association between circulating MP levels and stable CAD,[6,7] this is the first study that shows a relationship between MP procoagulant activity and plaque morphology in patients with proven, or suspected CAD.

Table 1. Baseline characteristics of the study population.

	NCP group (*n* = 76)	MCP group (*n* = 34)	CP group (*n* = 17)	*P*-value
Age, yrs	60 ± 12.7	67 ± 11.4	64 ± 10	0.021
Men, *n* (%)	41 (54)	26 (76)	14 (82)	0.014
BMI, kg/m^2	26.8 ± 3.8	27 ± 3.4	26 ± 1.98	0.65
Hypertension, *n* (%)	18 (24)	19 (56)	13 (76)	< 0.0001
Diabetes mellitus, *n* (%)	8 (11)	6 (18)	2 (12)	0.57
Hyperlipidemia, *n* (%)	42 (55)	23 (68)	15 (88)	0.06
Smoking, *n* (%)	19 (25)	2 (6)	1 (6)	0.02
Family history of CAD, *n* (%)	50 (66)	17 (50)	12 (70)	0.2
Previous myocardial infarction, *n* (%)	2 (2.6)	7 (21)	2 (12)	0.01
Stenosis ≥ 50%, *n* (%)	15 (20)	19 (56)	12 (70)	< 0.0001
Framingham risk score	13.9 ± 11	17.8 ± 10.4	19.4 ± 11	0.07
Laboratory profile				
Glucose, mg/dL	102.5 ± 11.5	102.0 ± 14.2	96.2 ± 22.7	0.24
Cholesterol, mg/dL	198.6 ± 44	185.5 ± 45.5	180.7 ± 42.2	0.18
LDL Cholesterol, mg/dL	127.3 ± 38.4	117.1 ± 47.9	115.3 ± 39.6	0.38
HDL Cholesterol, mg/dL	46.3 ± 14.3	48.2 ± 14.5	42.6 ± 12.3	0.43
Triglycerides, mg/dL	115 (86–151)	122 (85–141)	103 (83.5–130)	0.27
Creatinine, mg/dL	0.9 (0.8–1.08)	0.99 (0.8–1.1)	1.1 (0.9–1.9)	0.31
sLOX-1, pg/mL	1976 (686– 3027)	2226 (705–315)	1557 (272–2238)	0.43
CML, μg/mL	28 ± 8.2	31.8 ± 10	25.4 ± 6	0.03
MPs procoagulant activity, nmol/L	3.8 (2.4–8.0)	7.1 (3.7–9.7)	5.4 (3.3–7.5)	0.009

Data are given as mean ± SD, *n* (%) or median (interquartile range). *P*-values by ANOVA. BMI: body mass index; CAD: coronary artery disease; CP: calcified plaque; CML: N(epsilon)-(carboxymethyl) lysine; HDL: high density lipoprotein; LDL: low density lipoprotein; MCP: moderate calcified plaque; MPs: microparticles; NCP: non-calcified plaque; sLOX-1: soluble receptor for advanced glycation end-products.

Table 2. Vessel characteristics.

	Total patients	NCP group (n = 76)	MCP group (n = 34)	CP group (n = 17)
Vessel volume, mm^3	5530 ± 2411	5500 ± 2478	5420 ± 2374	5884 ± 2284
Total lumen volume, mm^3	3256 ± 1471	3358 ± 1572	3056 ± 1231	3207 ± 1486
Total plaque volume, mm^3	2274 ± 1239	2143 ± 1159	2364 ± 1392	2676 ± 1235
Total lumen volume, %	59.1 ± 10.6	61 ± 8.8	57.7 ± 12	54 ± 13*
Total plaque volume, %	40.4 ± 10.6	38.5 ± 8.8	41.9 ± 12.0	45.6 ± 13.1#

Data are given as mean ± SD. *P = 0.013 between CP group and NCP group; #P = 0.012 between CP group and NCP group. CP: calcified plaque; MCP: moderate calcified plaque; NCP: non-calcified plaque.

Table 3. Comparison of the MP procoagulant activity, CML and sLOX-1 levels among plaque groups.

	MP procoagulant activity (nmol/L)	CML (µg/mL)	sLOX-1 (pg/mL)
NCP group (n = 76)	3.8 (2.4–8.0)	28 ± 8.2	1976 (686–3027)
MCP group (n = 34)	7.1 (3.7– 9.7)*,#	31.8 ±10.0&,†	2226 (705–3151)
CP group (n = 17)	5.4 (3.3–7.5)	25.4 ± 6.0	1557 (272–2238)

Data are given as mean ± SD. *P = 0.01 between CP group and MCP group; #P = 0.005 between MCP group and NCP group; &P = 0.013 between CP group and MCP group; †P = 0.035 between MCP group and NCP group. CML: N (epsilon)-(carboxymethyl) lysine; CP: calcified plaque; MCP: moderate calcified plaque; MP: microparticles; NCP: non-calcified plaque group; sLOX-1: soluble form of lectin-like oxidized low-density lipoprotein receptor-1.

Atherosclerosis is a complex and multifactorial disease characterized by a cross-talk of cellular processes between the plaque and the blood. The basic pathology of adverse cardiovascular events, as consequences of CAD, involves not only the vulnerability of a plaque to rupture, but also a complex interplay of variables outside the plaque. In fact, changes in the systemic milieu can potentiate inflammation and thrombosis, characterizing a state of blood vulnerability that may induce plaque destabilization and rupture leading to dramatic cardiovascular events.

MPs generated within the blood compartment or at the blood-vessel interface play a key role in each step of the atherosclerosis, from early lesion formation to plaque instability and thrombus formation.[1] Our results suggest that the activation state of circulating MPs, which can also reflect an increase in MP concentration, may be of physiological significance, marking a cell-cell interaction resulting from endothelial dysfunction or platelet activation, that happen to
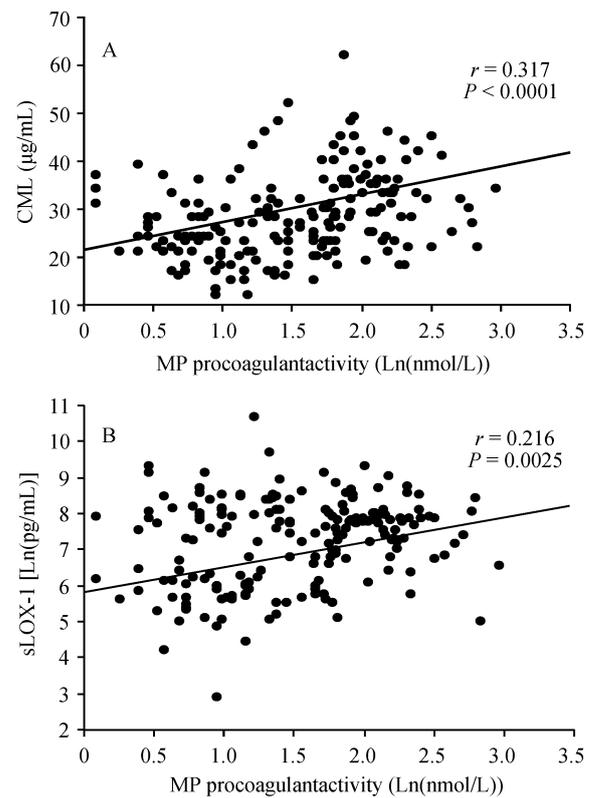
Figure 1. Pearson's correlation analysis of the MP procoagulant activity resulted significant in all patients. (A): Correlation between MP procoagulant activity and CML levels; (B): Correlation between MP procoagulant activity and sLOX-1 levels.

be associated with plaque progression. In fact, MPs released by dysfunctional endothelium can promote platelet activation and may at least be partly responsible for the prothrombotic state in patients with CAD.[21]

The composition of atherosclerotic plaque, including the size of lipid core, thickness of fibrous cap, number of macrophages and degree of calcification, rather than its volume, is the major determinant of averse atherosclerotic events in humans.[22] While stenotic plaques cause symptoms that prompt patents to seek treatment, vulnerable plaques tend to be asymptomatic and are often fatal.[23] Vulnerable plaques consist of a thin fibrous cap, marked with microcalcifications, over a lipid pool in the vessel wall. Calcification spots in the plaque fibrous cap may increase the vulnerability of plaque rupture because it alters the biomechanical stress on the fibrous cap.[24,25] Moderate calcification is associated with atheroma progression,[26] and is frequently observed in the culprit plaque of patients with acute coronary syndromes.[27,28] Evidence supports a central role for inflammation in all phases of the atherosclerotic process, from early atherosclerotic lesions, progressing to thrombotic complications of this disease.[29] Since the

plaque calcification process is triggered by oxidized lipids and pro-inflammatory cytokines,[30] a moderate calcified plaque may be characterized by high levels of inflammation, making it more prone to rupture. Inflammation and thrombosis are closely associated with one another, mutually contributing to altered circulating milieu inside the vessel.[31]

Lectin-like, oxidized, low-density lipoprotein receptor-1 (LOX-1) is linked to atherosclerotic plaque formation and the circulating form of this receptor may reflect activities of disease.[32] In patients with acute coronary syndrome, serum LOX-1 (sLOX-1) levels increased early,[32] and we and other authors showed that, in patients with stable CAD, sLOX-1 correlated with oxidative stress markers.[18,33] Recently, it has been shown that circulating sLOX-1 levels are associated with percutaneous coronary intervention-related periprocedural myocardial infarction, which might predict periprocedural myocardial necrosis in patients with stable CAD.[34] In our study, MP procoagulant activity is well correlated with sLOX-1 serum levels. Although in our cohort of patients the sLOX-1 levels did not differ among the three groups, and this association suggests that both markers might identify patients with a major pro-thrombotic/inflammatory status.

CML, the most commonly encountered advanced glycation end-product in vivo,[35] increases with aging and is enhanced in the vascular tissue of diabetic patients and in human atherosclerotic lesions.[36,37] Our findings show that CML levels were higher in the MCP patient group and that total CML levels significantly correlated with MP procoagulant activity. However, these results do not provide information on the mechanisms of the association of MPs with CML and SLOX-1 levels, nor if this association is a consequence of possible interactions among MPs, CML and SLOX-1 or the consequence of a common inflammatory burst.

4.1 Study limitations

Our study is a cross-sectional study, therefore cannot assign causality or mechanism to our findings. Another limit is the measure of coronary calcium content by CTA. The presence of contrast medium in CTA images forces the reduction of the window of HU for calcium recognition, causing an underestimation of calcium volume. However, data from our laboratory showed a strong correlation between coronary calcium content measured with standard calcium scoring method without contrast medium and CTA images ($Y = 1.47 + 0.128 \times X$; R2 = 0.874, F = 660.6, $P < 0.00001$). Avoiding a dedicated CT acquisition for calcium scoring before the angiographic studies allowed about a 10% sparing of radiation dose. Finally, MPs were detected by using a microplate affinity capture assay, which detects only MPs that expose functional phosphatidyl-serine on

their surface and does not provide information about their size and cellular origin.

4.2 Conclusions

Our findings suggest that the procoagulant nature of MPs in the MCP group might contribute to progression of atherosclerotic plaque and lead to deleterious clinical events if a thrombogenic state is present at the time of plaque fissuring or rupture. Thus, the measurement of MP in CAD patients could be a useful tool for identifying subjects at high risk of adverse cardiac events.

Acknowledgements

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