Circulating Leukocyte-Derived Microparticles Predict Subclinical Atherosclerosis Burden in Asymptomatic Subjects

Gilles Chironi, Alain Simon, Bénédicte Hugel, Muriel Del Pino, Jérôme Gariepy, Jean-Marie Freyssinet, Alain Tedgui

Objective—To clarify circulating microparticles (MP) relationships with preclinical atherosclerosis.

Methods and Results—In 216 subjects without cardiovascular disease, we assessed: (1) annexin V-positive, platelet-derived, endothelium-derived and leukocyte-derived circulating MP by capture on annexin V, anti-GPIb, anti-CD105, and anti-CD11a antibody-coated wells, respectively; (2) Framingham risk, metabolic syndrome, and low-grade inflammation by risk factors measurement including hsCRP; and (3) subclinical atherosclerosis by ultrasound examination of carotid, abdominal aorta, and femoral arteries. Number of sites with plaque ranged from 0 to 3 and plaque burden was classified into 0 to 1 or 2 to 3 sites disease. Leukocyte-derived MP level was higher in the presence than in the absence of moderate to high Framingham risk \( (P<0.05) \), metabolic syndrome \( (P<0.01) \), high C-reactive protein (CRP) \( (P<0.05) \), or 2- to 3-sites disease \( (P<0.01) \), and correlated positively with number of metabolic syndrome components \( (P<0.001) \), tertiles of fibrinogen \( (P<0.001) \), and number of diseased sites \( (P<0.01) \). In multivariate analysis, 2- to 3-sites disease was independently associated with leukocyte-derived MP level \( (P<0.05) \), Framingham risk \( (P<0.01) \), and metabolic syndrome \( (P<0.01) \). None of the other MP types correlated with risk markers or atherosclerosis.

Conclusions—Leukocyte-derived MP, identified by affinity for CD11a, are increased in subjects with ultrasound evidence of subclinical atherosclerosis, unveiling new directions for atherosclerosis research. (Arterioscler Thromb Vasc Biol. 2006;26:2775-2780.)

Key Words: atherosclerosis ■ leukocytes ■ microparticles ■ risk factors ■ ultrasonic diagnosis

Shed membrane microparticles (MP) in peripheral blood originate from diverse apoptotic or activated cells of endothelial, leukocyte, and platelet type and are involved in the pathogenesis of atherothrombotic disease.\(^1\)\(^-\)\(^3\) Indeed, MP isolated from blood of patients with clinically overt cardiovascular disease can initiate thrombosis and cause endothelial dysfunction in vitro and in experimental vascular models.\(^4\)\(^-\)\(^5\) Also, the number of circulating MP of endothelial and platelet origin is increased in patients with acute coronary syndromes\(^6\) or chronic kidney failure with vasculopathy.\(^7\) Nevertheless, little is still known about the potential participation of circulating MP to early stage of arterial disease before complication has occurred.\(^1\)\(^-\)\(^3\)\(^,\)\(^8\)\(^-\)\(^9\) To clarify this question we measured circulating MP level in symptom-free subjects without known history of cardiovascular disease and explored their potential relationships with cardiovascular risk factors and subclinical atherosclerosis assessed by ultrasound.\(^10\)\(^-\)\(^12\)

Methods

Subjects

The study population was constituted of consecutive asymptomatic subjects addressed between December 2004 and July 2005 to our Center of Cardiovascular Prevention by occupational health medicine doctors in the framework of a program of cardiovascular prevention described previously,\(^10\)\(^-\)\(^13\) aiming to determine their cardiovascular risk factor profile. A condition of selection was that they were free of any cardiovascular disease (stroke, coronary heart disease, heart failure, peripheral vascular disease, and diabetes), and that they had undergone measurement of circulating MP after written informed consent. Body mass index was calculated as the ratio of weight-to-height squared and waist circumference was measured with a tape horizontal around the abdomen at the level of the right iliac crest.\(^14\)\(^,\)\(^15\) Resting brachial blood pressure was measured by sphygmomanometer, and hypertension was defined as blood pressure \( \geq 140/90 \text{ mm Hg} \) and/or the presence of antihypertensive treatment.\(^16\) Fasting blood lipids and glucose were measured by enzymatic methods (after precipitation of low-density lipoprotein...
[LDL] and very-low-density lipoprotein [VLDL] for high-density lipoprotein [HDL] measurement), and hypercholesterolemia was defined by total cholesterol of ≥5.2 mmol/L, and/or the presence of lipid lowering drug treatment. Plasma high-sensitivity C-reactive protein (CRP) was measured by immunoassay and plasma fibrinogen was measured by the Clauss method. Current smoking was defined by daily consumption of ≥1 cigarette for ≥3 months. Framingham risk of coronary heart disease (% at 10 years) was estimated by entering age, male sex, systolic pressure, total and HDL cholesterol, and presence or absence of smoking into the Framingham Model equations, and study subjects were classified as low risk (<10%) or moderate to high risk (≥10%). Metabolic syndrome was considered present when 3 of the following 5 criteria were present: (1) waist circumference >94 cm in men or >80 cm in women, in accordance with cut-offs for European subjects; (2) triglycerides >1.71 mmol/L; (3) HDL cholesterol <1.03 mmol/L in men or <1.29 mmol/L in women; (4) blood pressure ≥130/85 mm Hg and/or on antihypertensive medication; and (5) fasting blood glucose ≥5.6 mmol/L. Inflammatory risk was graded according to three following levels of CRP: low CRP as <1 mg/L, intermediate CRP as ≥1 and <3 mg/L, and high CRP as ≥3 mg/L.

Circulating MP
MP were isolated from platelet-poor plasma and captured by immobilized specific anti-GPIb, anti-CD105 or anti-CD11a antibodies as previously described. The procoagulant phospholipid content was determined by a prothrombinase assay and the amount of MP (MP level) was expressed as nanomolar phosphatidylserine (PS) equivalent.

MP Sample Preparation
All samples were prepared and stored as follows. Five milliliters of 12-hour fasting venous blood were withdrawn on citrated tube. Within the 2 hours after withdrawal, samples were centrifuged at 1500g for 15 minutes at room temperature, and the supernatant was centrifuged again at 13 000g for 2 minutes to avoid platelet contamination. The so-obtained platelet-poor plasma was distributed into 4 aliquots of 500 μL each, frozen, and stored at -80°C for a maximum of 2 months. After thawing, thrombin and factor Xa inhibitors, respectively, D-phenylalanyl-prolyl-arginyl chloromethyl ketone (APA, Bachem, Philadelphia, PA), and 1,5-dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (1,5D-GaMK, Bachem, Philadelphia, PA) were added to plasma samples at a final concentration of 50 μmol/L each and CaCl₂ at a final concentration of 50 mmol/L.

Circulating Procoagulant MP Quantitation
After capture of MP on annexin V-coated wells (for 30 minutes at 37°C), taking advantage of the strong affinity of annexin V for phosphatidylserine, MP were isolated from platelet-poor plasma and captured by immobilized specific antibodies to GPIb, anti-CD105 or anti-CD11a antibodies as previously described. The procoagulant phospholipid content was determined by a prothrombinase assay and the amount of MP (MP level) was expressed as nanomolar phosphatidylserine (PS) equivalent.

Statistical Analysis
Data are presented as means (SD) or number of subjects n (%).
transformed MP levels were compared between groups by ANOVA and additional nonparametric tests (Wilcoxon) were performed without log-transformation of MP. Associations between log-transformed MP and continuous parameters were analyzed by linear regression by least square method. Proportions of subjects with 2 to 3 sites disease were compared between subgroups by \textit{H} test.

Independent associations of MP with atherosclerosis were tested by logistic regression entering 2- to 3-sites disease as dependent variable, and log-transformed MP level and covariates as independent variables. Statistical significance was set at \textit{P}<0.05.

\textbf{Results}

\textbf{Subjects Characteristics}

Two hundred sixteen subjects were eligible for the study. There was a high prevalence of hypertension (40%), hypercholesterolemia (39%), and smoking (23%). The presence of moderate to high Framingham risk was found in 44% of patients, that of metabolic syndrome in 37%, and high level of CRP in 18%. Approximately half of the subjects had 2- to 3-sites disease. Subjects characteristics are detailed in Tables 1 and 2.

\textbf{MP and Circulating Blood Cells}

Leukocyte-derived MP level was positively associated with leukocyte cell count (\textit{r}=0.52, \textit{P}<0.001). Annexin V-positive and platelet-derived MP levels were positively associated with platelet count (\textit{r}=0.23, \textit{r}=0.24 respectively, \textit{P}<0.001). None of the MP categories were associated with hematocrit.

\textbf{MP and Cardiovascular Risk}

No significant difference existed in annexin V-positive, platelet-derived and endothelium-derived MP levels between subjects with moderate to high Framingham risk and those with low Framingham risk, or between subjects with metabolic syndrome and those without, or between subjects with high CRP and those with low or intermediate CRP (Table 2). In contrast, leukocyte-derived MP level was higher in subjects with moderate to high Framingham risk than in low Framingham risk subjects (\textit{P}<0.05) and in subjects with metabolic syndrome than in those without (\textit{P}=0.001) (Table 2). Also, leukocyte-derived MP level differed between the 3 grades of CRP (\textit{P}<0.05), with higher value in subjects with high CRP than in those with low CRP (\textit{P}<0.01) (Table 2). It is noteworthy that similar levels of statistical significance were obtained by using Wilcoxon test without log-transformation of MP (respectively, \textit{P}<0.05, \textit{P}<0.001, \textit{P}<0.05).

\textbf{MP and Subclinical Atherosclerosis}

No significant difference in annexin V-positive, platelet-derived and endothelium-derived MP levels existed between subjects with 2- to 3-sites disease and those with 0- to 1-site disease (Table 2). In contrast, leukocyte-derived MP level

\begin{table}[h]
\centering
\caption{Circulating Procoagulant (Annexin V), Platelet-Derived (GPIb), Endothelium-Derived (CD105), and Leukocyte-Derived (CD11a) MP Levels, by Degree of Cardiovascular Risk Markers and Atherosclerotic Plaque Burden}
\begin{tabular}{lcccc}
\hline
 & \textbf{n} & \textbf{Annexin V} & \textbf{GPIb} & \textbf{CD105} & \textbf{CD11a} \\
\hline
\textbf{Framingham risk} & & & & & \\
Low & 121 & 4.91 (2.54) & 3.01 (2.61) & 0.42 (0.19) & 3.18 (1.39) \\
Moderate to high & 95 & 4.92 (3.32) & 3.25 (2.81) & 0.47 (0.17) & 3.53 (1.53) \\
\textbf{P} & & 0.988 & 0.476 & 0.183 & 0.048 \\
\textbf{Metabolic syndrome} & & & & & \\
Absent & 136 & 4.99 (2.53) & 3.02 (2.77) & 0.43 (0.20) & 3.12 (1.36) \\
Present & 80 & 4.79 (3.46) & 3.27 (2.57) & 0.46 (0.15) & 3.69 (1.57) \\
\textbf{P} & & 0.632 & 0.248 & 0.346 & 0.001 \\
\textbf{C-reactive protein} & & & & & \\
Low & 100 & 4.81 (2.48) & 2.70 (2.03) & 0.43 (0.18) & 3.07 (1.24) \\
Intermediate & 78 & 5.32 (3.70) & 3.33 (3.00) & 0.44 (0.18) & 3.43 (1.38) \\
High & 38 & 4.45 (2.09) & 3.91 (3.46) & 0.48 (0.18) & 3.85 (2.02)* \\
\textbf{P} & & 0.281 & 0.141 & 0.299 & 0.016 \\
\textbf{Plaque burden} & & & & & \\
0- to 1-site disease & 104 & 5.10 (3.20) & 3.11 (2.83) & 0.43 (0.18) & 3.08 (1.21) \\
2- to 3-sites disease & 112 & 4.72 (2.54) & 3.12 (2.56) & 0.45 (0.19) & 3.59 (1.66) \\
\textbf{P} & & 0.337 & 0.734 & 0.512 & 0.006 \\
Normal ranges† & 40 & 4.92 (1.98) & 3.07 (2.17) & 0.42 (0.21) & 3.08 (1.43) \\
\hline
\end{tabular}
\\textit{Data are expressed as means (SD); n, number of subjects. \textit{P} values are obtained by ANOVA after log-transformation of MP levels.}
\textit{*P}<0.01 as compared with low CRP.
\textit{†Unpublished data in normal healthy volunteers.}
\end{table}
was higher in subjects with 2- to 3-sites disease than in those with 0- to 1-site disease ($P<0.01$; Table 2), with a similar difference obtained by using Wilcoxon test without log-transformation of MP ($P<0.05$). A positive relationship existed between leukocyte-derived MP level and the number of sites with presence of plaque ($r=0.20$, $P<0.01$; Figure 1). When study subjects were divided into 4 subsets based on leukocyte-derived MP level (low, $<$, or high, $\geq$, median value, 3.3 nmol/L PS) and Framingham risk score (low, $<$, or high, $\geq$, median value, 8.6%), the prevalence of 2- to 3-sites disease was greatest (70%) in individuals with high Framingham risk score and high leukocyte-derived MP level, and lowest (27%) in those with low Framingham risk score and low leukocyte-derived MP level ($P<0.001$; Figure 2). Multivariate regression models, adjusted for leukocyte cell count and coexisting antihypertensive and lipid-lowering therapy, showed that the presence of 2- to 3-sites disease was independently associated with leukocyte-derived MP level ($P<0.05$), Framingham risk score ($P<0.001$), and presence of metabolic syndrome ($P<0.01$), but not CRP (model 1) or fibrinogen (model 2) (Table 3).

### Discussion

The present investigation clearly evidenced a relationship between the levels of circulating leukocyte-derived MP and the degree of cardiovascular risk markers and atherosclerotic plaque burden. It has to be emphasized that MP values of our low-risk low-inflammation subgroups without atherosclerosis were not different from those observed in healthy individuals in our routine practice (unpublished data, Table 2).

The involvement of leukocyte-derived MP in atherosclerosis has been previously reported in symptomatic patients with clinically overt arterial disease, whose atherosclerotic plaques contained great amounts of leukocyte-derived MP, but their participation to the preclinical early stage of atherosclerotic disease is suggested for the first time by the present study. Ultrasound interrogation of 3 arterial sites, carotid arteries, abdominal aorta, and femoral arteries, in a cohort of asymptomatic subjects without cardiovascular disease, allowed us to find that leukocyte-derived MP level, measured by CD11a antibody in peripheral blood, was higher in subjects carriers of atherosclerotic plaque in 2 or 3 sites than in those without plaque at any site or with plaque in only 1 site. Moreover, circulating leukocyte-derived MP level increased significantly, although weakly, gradually in function of the presence of plaque in 0, 1, 2 or 3 sites. The positive association between leukocyte-derived MP level and presence of high degree of plaque burden (2 to 3 sites disease) was independent of coexisting cardiovascular risk factors, circulating leukocyte cell count and coexisting anti-hypertensive or lipid-lowering drugs. These findings suggest some etiologic link between leukocyte-derived MP and the burden of subclinical atherosclerosis within the arterial tree. They also allow to consider leukocyte-derived MP as potential valuable predictors for subclinical atherosclerosis burden, with a predictive value, estimated by odds ratio of having 2- to 3-sites disease in multivariate regression analysis, similar to that of metabolic syndrome and CRP but half lesser than that of Framingham risk score. Anyway, as illustrated by Figure 2, leukocyte-derived MP level adds a useful predictive value to that of Framingham risk score as regards the risk of diffuse atherosclerosis. The link of leukocyte-derived MP with plaque burden being independent of conventional cardiovascular risk factors, specific deleterious effects of leukocyte-derived MP on the arterial wall are likely to occur. One possible effect, supported by a study of circulating leukocyte-derived MP in normal volunteers, may be to stimulate the release of inflammatory cytokines and the expression of functional tissue factor in endothelial cells, that may potentially contribute to endothelial dysfunction. Another effect, reported in mouse and concerning MP derived from T lymphocytes, may be to induce dysregulation of endothelial function of conductance arteries by affecting nitric oxide synthase.
Circulating leukocyte-derived MP level was also influenced by various cardiovascular risk markers such as metabolic syndrome, low-grade inflammation, or Framingham risk score, independently of its relationship with atherosclerotic plaque burden. Although the associations between leukocyte-derived MP level and atherosclerotic risk factors were somewhat weak, they remained statistically significant whether data were analyzed by using log transformation or parametric testing. In subjects with metabolic syndrome, leukocyte-derived MP level was higher than in those free of such syndrome and in the overall study population, leukocyte-derived MP level increased gradually in parallel with the number of components of metabolic syndrome. These findings agree with previous studies showing that type 2 diabetic patients present an increased number of circulating MPs that are the preponderant reflection of platelet-derived MP. Moreover, the lack of association of endothelium-derived MP level with cardiovascular risk or preclinical atherosclerosis may be caused by the weak endothelial injury and apoptosis in our asymptomatic subjects at early stage of arterial disease. However, it cannot be excluded that the discrepancies between our present data and previous studies showing increased endothelial-derived MP level in various clinical conditions result from methodological differences between the cited studies and ours. In one case, endothelial markers used for immunocapture assay were different from those used in our study; in others, MPs were detected by fluorescence activated cell sorter (FACS) analysis instead of immunocapture assay. This explanation applies also to circulating procoagulant MP that are the preponderant reflection of platelet-derived MP. Further studies using different approaches to detect MP, including FACS analysis instead of solid-phase capture assays, are required to address this possibility. Also, we cannot be ruled out that some annexin V-negative MP contribute to increased atherosclerotic risk. Further studies using different approaches to detect MP, including FACS analysis, are required to address this possibility. Also, we cannot be ruled out that some annexin V-negative MP contribute to increased atherosclerotic risk. Further studies using different approaches to detect MP, including FACS analysis, are required to address this possibility.

### Study Limitations

MPS were detected by using microplate affinity capture assays, which do not provide information about MP size and plaque burden, Framingham risk, metabolic syndrome, or low-grade inflammation constitutes a last series of findings that merits discussion. They are discrepant with the results of previous studies in patients with clinically advanced arterial disease showing that myocardial infarction, coronary artery disease, or end-stage renal failure were associated with increased number of endothelium and/or platelet-derived MP. They also contradict a previous observation that the number of endothelium-derived MP was positively related with most traditional cardiovascular risk factors in patients with coronary artery disease. Such discrepancies may be explained by the fact that platelet-derived MP exert their major effect on thrombogenesis, which is absent or minimal at the preclinical stage of arterial disease before atherothrombotic complication. This explanation applies also to circulating procoagulant MP that are the preponderant reflection of platelet-derived MP. Moreover, the lack of association of endothelium-derived MP level with cardiovascular risk or preclinical atherosclerosis may be caused by the weak endothelial injury and apoptosis in our asymptomatic subjects at early stage of arterial disease. However, it cannot be excluded that the discrepancies between our present data and previous studies showing increased endothelial-derived MP level in various clinical conditions result from methodological differences between the cited studies and ours. In one case, endothelial markers used for immunocapture assay were different from those used in our study; in others, MPs were detected by fluorescence activated cell sorter (FACS) analysis instead of immunocapture assay. Other studies in asymptomatic subjects have shown that endothelial-derived MPs were increased, either acutely after a single ingestion of fatty meal, or chronically in presence of metabolic syndrome. This discrepancy with our findings could again arise from different assay system, because the mentioned studies used FACS analysis instead of solid-phase capture assays.

### Study Limitations

MPS were detected by using microplate affinity capture assays, which do not provide information about MP size and detect only MP which expose functional PS on their surface, depending on activation versus apoptosis. As a result, it cannot be ruled out that some annexin V-negative MP contribute to increased atherosclerotic risk. Further studies using different approaches to detect MP, including FACS analysis, are required to address this possibility.

### Table 3: Logistic Regression of the Presence of 2- to 3-Sites Disease on Circulating Leukocyte-Derived MP Level and Cardiovascular Risk Markers Including Either C-Reactive Protein (Model 1) or Plasma Fibrinogen (Model 2), With Additional Adjustments for Leukocyte Cell Count and Coexisting Antihypertensive and Lipid-Lowering Treatments

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR [95% CI]</td>
<td>P</td>
<td>OR [95% CI]</td>
<td>P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MP level, nmol/L PS</td>
<td>1.45 [1.04–2.06]</td>
<td>&lt;0.05</td>
<td>1.43 [1.02–2.05]</td>
<td>&lt;0.05</td>
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</tr>
<tr>
<td>Framingham risk, %</td>
<td>3.07 [2.02–4.89]</td>
<td>&lt;0.001</td>
<td>3.22 [2.12–5.13]</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>Metabolic syndrome, presence</td>
<td>1.38 [1.12–1.73]</td>
<td>&lt;0.01</td>
<td>1.35 [1.10–1.69]</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>C-reactive protein, mg/L</td>
<td>1.39 [0.99–1.98]</td>
<td>0.06</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Plasma fibrinogen, g/L</td>
<td>—</td>
<td>—</td>
<td>1.18 [0.84–1.23]</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.23</td>
<td>—</td>
<td>0.22</td>
<td>—</td>
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</tbody>
</table>

Data are odds ratios (OR) with 95% confidence interval (CI) of having 2- to 3-sites disease by 1SD increase in continuous independent variable or by presence of qualitative independent variable. p values are calculated after log-transformation of MP level and CRP. NS indicates nonsignificant.
cannot exclude that a lack of sensitivity of MP markers may be a limitation for analyzing our asymptomatic subjects. This may contribute to the lack of relationship between platelet- and endothelial-derived MP and atherosclerotic risk markers.

In conclusion, circulating leukocyte-derived MP are independently associated with subclinical atherosclerosis burden, and this may unveil an important new direction for atherosclerosis research.

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Disclosures

None.

References


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