Biomarkers in ischemic cardiac syndromes

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Citation for published version (APA):
van der Zee, P. M. (2010). Biomarkers in ischemic cardiac syndromes.
Chapter 2

P-selectin- and CD63-exposing platelet microparticles reflect platelet activation in peripheral arterial disease and myocardial infarction

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Clinical Chemistry 2006;52:657-64.
Abstract

Objective. Platelet-derived microparticles (PMP) are generally considered a marker of platelet activation in cardiovascular disease. We studied to which extent subpopulations of PMP parallel platelet activation in vitro and in vivo.

Methods. Using flow cytometry, we analyzed PMP subpopulations from resting and activated platelets in vitro (n = 6) as well as from plasma samples of patients with stable angina, peripheral arterial disease, or myocardial infarction [non-ST-elevation (NSTEMI) and ST-elevation (STEMI)] and from older, age- and sexmatched and young healthy individuals [n = 10 for all groups except NSTEMI (n = 11)]. Coagulation markers prothrombin fragment F$_{1+2}$ and thrombin-antithrombin complexes were determined by ELISA. The PMP-associated fraction of soluble (s)P-selectin was estimated by ELISA.

Results. In vitro, stimulation of platelets with thrombin receptor–activating peptide (15 µmol/L) or the calcium ionophore A23187 (2.5 µmol/L) increased fractions of both platelets and PMPs exposing P-selectin or CD63 (P <0.001 for all). Whereas the number of PMPs released by A23187-stimulated platelets increased significantly (P <0.001), the number of PMPs released from thrombin receptor-activating peptide–stimulated platelets remained constant (P >0.05). Ex vivo, numbers of circulating PMPs were comparable in all groups. Compared with young persons, P-selectin–exposing PMPs were increased in older persons (P = 0.02) and were further increased in patients with NSTEMI (P = 0.007) and STEMI (P = 0.045). CD63-exposing PMPs were increased in patients with peripheral arterial disease (P = 0.041), NSTEMI (P = 0.001), and STEMI (P = 0.049). Subpopulations exposing P-selectin or CD63 correlated with each other (r = 0.581; P <0.001), but neither correlated with the plasma concentrations of F$_{1+2}$ or thrombin–antithrombin complexes. The PMP-associated fraction of sP-selectin constituted only 2.2 (4.7)% [mean (SD)] of total sP-selectin.

Conclusions. PMP subpopulations reflect platelet activation status better than the total number of PMPs. Increased concentrations of circulating PMP subpopulations are found in aging, and further increases are encountered in peripheral arterial disease and myocardial infarction.
Introduction

Platelet activation occurs during development of atherosclerosis as well as thrombus formation in acute manifestations of atherosclerotic disease (1). Accurate determination of their activation status remains a challenge, but could provide a useful tool in identifying patients at risk for future cardiovascular events.

Current methods are based on the detection of reversible and irreversible platelet activation that can be monitored by direct and indirect methods (2). Direct methods include flowcytometric analysis of platelet membrane glycoproteins that, on activation, either become exposed (e.g. P-selectin or CD63) or change conformation (e.g. glycoprotein IIb-IIIa), as well as measurements of platelet secretion products in plasma or urine. Whereas platelet flowcytometry requires fresh samples, is labor intensive and complicated by fixation procedures, secretion products are either not very specific for platelets (e.g. soluble P-selectin) or are sensitive to sampling and handling artefacts (e.g. thromboglobulin).

Indirect methods are functional platelet assays, which evaluate the ex vivo platelet function which is thought to be dependent on the platelet activation status in vivo. In this way, both reversible (e.g. aggregation and adhesion) and irreversible platelet activation (i.e., the secretion response), can be studied. Although rapid platelet function assays have been developed, correlations with direct assays are poor, suggesting that such functional assays do not necessarily reflect the actual in vivo activation status (3).

In vitro, the release of microparticles (MP) from platelets (platelet-derived MP; PMP) has been associated with the secretion response (4). Therefore, numbers of circulating PMP are considered to reflect the platelet activation status in vivo, for example, in patients with peripheral arterial disease (5), unstable angina (6), myocardial infarction (MI) (7), cerebrovascular accident (8, 9) and diabetes (9). Because analysis of PMP does not require fixation and can be applied to stored plasma samples, batchwise analysis of the in vivo platelet activation status is feasible. However, although several studies confirmed concomitant changes in exposure of platelet P-selectin and circulating numbers of PMP in vivo (10, 11), others found discrepancies between both (6, 7, 12-14), which may question the relevance of PMP numbers as a marker of platelet activation.

We hypothesized that measuring PMP-associated P-selectin or CD63 is a feasible and reliable method of assessing platelet activation. Because of the intricacy of adequately assessing the in vivo platelet activation status as discussed before, we first evaluated the exposure of P-selectin and CD63 on both platelets and PMP under well controlled in vitro conditions. To substantiate the relevance of our in vitro findings, we also determined the exposure of P-selectin and CD63 on circulating PMP in subject groups with various degrees of atherosclerotic disease. Finally, we measured the coagulation activation markers prothrombin fragment F1+2 and thrombin-antithrombin complexes (TAT), which have been associated with platelet activation and PMP release (15).
Materials and methods

Reagents and assays
For detection of MP origin, we used phycoerythrin (PE)-labeled antiglycophorin A (JC159, IgG1) and fluorescein isothiocyanate (FITC)-labeled anti-CD61 (Y2/51, IgG1) (Dako A/S, Glostrup, Denmark); anti-CD4-PE (CLB-T4/2,6D10, IgG1), and anti-CD66e-PE (CLB-gran/10, IH4Fc, IgG1) Sanquin, Amsterdam, the Netherlands, anti-CD8-PE (SK1, IgG1), anti-CD14-PE (MØP9, IgG2a), anti-CD20-PE (L27, IgG1), IgG1-FITC (X40), and IgG1-PE (X40) from BD (San José, CA), anti-CD62e-PE (HAE-1f, IgG1) from Ancell Corp. (Bayport, MN), anti-CD54-PE (84H10, IgG1), allophycocyanin (APC)-labeled annexin V from Caltag (Burlingame, CA), IgG2a-PE (MCG2b) from Immuno Quality Products (Groningen, the Netherlands), anti-CD63-PE (CLB-gran12, IgG1) and anti-P-selectin-PE (CLB-Thromb/6, IgG1) from Coulter/Immunotech (Marseille, France). Previously we demonstrated that the anti-P-selectin antibody used does not cross-react with (endothelial) E-selectin (16). Plasma concentrations of sP-selectin, F1+2 and thrombin-antithrombin complexes (TAT) were determined ELISA as described by the manufacturer (Parameter human P-Selectin Immunoassay by R&D Systems, Minneapolis, MN; Enzygnost F1+2 micro and TAT by Dade-Behring Diagnostics GmbH, Germany, respectively).

In vitro platelet activation and flow cytometric analysis
We obtained venous blood from 6 healthy individuals who had not taken any medication during the previous 10 days and had given their informed consent. The blood was collected into 1/10th volume of 10.9 mmol/L trisodium citrate and was centrifuged at 180g for 15 min at 20 °C to obtain platelet-rich plasma (PRP). PRP (3.5– 4.5 mL) was acidified with 1/6th volume of acid-citrate-dextrose (71 mmol/L citric acid, 85 mmol/L trisodium citrate, and 110 mmol/L d-glucose, pH = 4.4). The acidified PRP from each healthy individual was divided into 2 aliquots. We added dibutyryl cAMP (final concentration, 2 mmol/L) to one of the aliquots; to the other we added nothing. The PRP was incubated for 20 min at 37 °C. Platelets were then pelleted by centrifugation at 700g for 20 min at 20 °C, then washed once with a buffer containing 137 mmol/L NaCl, 2.6 mmol/L KCl, 1.0 mmol/L MgCl2, 11.9 mmol/L NaHCO3, 5.6 mmol/L d-glucose, 1 mmol/L EDTA (buffer A; pH 6.5). For the platelet suspensions pretreated with dibutyryl cAMP, this buffer also contained 2 mmol/L dibutyryl cAMP. Finally, platelets were resuspended in buffer A without EDTA or dibutyryl cAMP (4.5 mL; pH 7.35). Platelet counts were determined with a CELL-DYN 4000 hematology analyzer (Abbott Diagnostics Division, Abbott Laboratories).
We added nothing to 1-mL aliquots of the platelet suspensions treated with dibutyryl cAMP (nonactivated platelets). To 1-mL aliquots of the platelet suspensions not treated with dibutyryl cAMP, we added CaCl2 (final concentration, 2.5 mmol/L) alone, as a control, or in combination with either thrombin receptor–activating peptide [(TRAP); FLLRN from Bachem AG; final
concentration, 15 µmol/L] or the calcium ionophore A23187 (Calbiochem; final concentration, 2.5 µmol/L). The mixtures were incubated at 37 °C for 30 min without stirring to avoid platelet aggregation. Activation was stopped by the addition of 2.5 mmol/L (final concentration) of EDTA, and platelet counts were determined. For flow cytometric analysis, 5-µL aliquots were taken from each tube and diluted in 35 µL of HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1 mmol/L MgCl2, 20 mmol/L HEPES, 3.3 mmol/L NaH2PO4, 1 g/L bovine serum albumin, 5.6 mmol/L d-glucose; pH 7.4). Anti-CD61-FITC and a PE-labeled anti-P-selectin (CD62p) or anti-CD63 monoclonal antibody (5 µL each) were added. The mixtures were incubated in the dark for 30 min at 20 °C, after which the platelets were fixed by addition of 1.5 mL of 3 g/L paraformaldehyde and analyzed on a FACS Calibur flow cytometer with CellQuest Pro 4.0.2 software (BD Diagnostics). Forward scatter (FSC), side scatter (SSC), and fluorescence data were obtained with gain settings in the logarithmic mode. Platelets and PMPs were selected based on their platelet marker positivity and FSC/SSC characteristics (17,18). For each sample, 10 000 platelet marker–positive (CD61) events were acquired. To identify marker-positive events, thresholds were set based on samples incubated with similar concentrations of isotype-matched control antibodies.

**Patient and healthy control groups**

Blood samples were obtained from (a) patients with stable angina pectoris (Canadian Society of Cardiology class II) having at least 1 significant (>50%) coronary stenosis (n = 10); (b) patients with ST-elevation myocardial infarction (STEMI; n = 10) (19); (c) patients with non-STEMI (NSTEMI; n = 11) (19); and (d) patients with angiographically confirmed peripheral arterial disease (n = 10). We collected blood samples from cardiac patients at the Department of Cardiology of the Academic Medical Center before they underwent catheterization and/or percutaneous coronary intervention. Blood samples from patients with peripheral arterial disease were obtained at the Department of Vascular Surgery before patients underwent elective bypass surgery, and patients were advised to refrain from strenuous exercise at least 24 h before sampling. All NSTEMI patients were sampled within 12 h and all STEMI patients within 6 h after onset of symptoms. All patients received medication according to standard clinical practice. None received coumarin derivatives, thrombolytic therapy, or antiplatelet medication other than aspirin. Patients with stable angina pectoris received aspirin therapy, whereas those with peripheral arterial disease were advised to refrain from aspirin use for at least 7 days before elective surgery and received prophylactic low–molecular-weight heparin. Patients with MI had received aspirin and low–molecular-weight heparin on admission. Blood samples were also obtained from healthy individuals, age- and sex-matched to the patient groups (n = 10), as well as from healthy individuals <50 years of age (n = 10). The study was approved by the local medical ethics committee of the Academic Medical Center. Venous blood was collected from the cubital vein.
into 1/10th volume of 10.9 mmol/L trisodium citrate (BD Diagnostics) with minimal venous occlusion. All blood samples were collected by a single clinician (P.M.v.d.Zee) and processed within 30 min. Blood samples were centrifuged (20 min at 1550g) at room temperature. Only the upper two-thirds of the apparent plasma fraction were collected and briefly mixed in a plastic 10-mL tube. Aliquots of 250 µL were immediately snap-frozen in liquid nitrogen for at least 15 min, to be finally stored at -80 °C until assay.

MP isolation and flow cytometric analysis

We isolated MPs as described previously (17,18). Briefly, we centrifuged snap-frozen plasma samples (250 µL) at 20 °C for 30 min at 17 570g. The upper 225 µL were discarded. The remaining plasma was diluted with 225 µL of phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4) containing 10.9 mmol/L trisodium citrate (PBS/citrate buffer). MPs were resuspended and centrifuged again (30 min at 17 570g and 20 °C). After removal of the supernatant (225 µL), MPs were resuspended and diluted 4-fold in PBS/citrate buffer. We used 5-µL aliquots of this suspension for flow cytometric analysis. Samples were analyzed in a FACSCalibur with CellQuest software (BD Diagnostics) (18). We double-stained MP samples from 6 patients in each group with annexin V and a cell-specific monoclonal antibody; we triple-stained MPs from 10 patients in each group with annexin V, anti-CD61, and either anti-P-selectin or anti-CD63. MPs were analyzed for 1 min and identified by their characteristic FSC and SSC and by their ability to bind annexin V and a cell-specific monoclonal antibody (17,18,20). From the number of events (N) in the upper right (marker- and annexin V-positive) quadrant of the flow cytometric analysis (FL-1 vs FL-2, corrected for isotype control antibody binding and autofluorescence), the number of MPs per liter of plasma was calculated as:

\[ n/L = N \times (100/5) \times (955/60) \times (10^6 / 250), \]

in which 5 (µL) is the volume of MP suspension, 100 is the total volume of washed MP suspension, 955 is the total volume in the tube before analysis, 60 is the sample volume analyzed, 106 is the number of microliters per liter, and 250 is the original volume of plasma.

Statistical analysis

All data not gaussian distributed are presented as the median (interquartile range); other data are presented as the mean (SD). t-Tests and Mann–Whitney U-tests were used for comparisons of gaussian- and nongaussian distributed data, respectively. The \( \chi^2 \) test was used for comparison of dichotomous variables. For multiple comparisons, the Kruskal–Wallis test or one-way ANOVA for matched samples was used, followed by the Bonferroni multiple comparison test. Correlations were calculated in patients and healthy persons together, with the Spearman rank correlation test. P < 0.05 was regarded as statistically significant.
Results

Washed human platelets (n=6) were stimulated with either TRAP (15 μM) or the calcium ionophore A23187 (2.5 μM). As shown in figure 1A, compared with nonactivated (i.e., dibutyryl cAMP-treated) platelets, the fractions of platelets exposing P-selectin (CD62p) or CD63 increased strongly in response to both TRAP and A23187 (P <0.001 for both P-selectin and CD63, using either agonist). Platelet disappearance during stimulation was negligible (P >0.05 compared with nonactivated platelets; data not shown). The fractions of PMPs exposing P-selectin or CD63 in response to TRAP or A23187 are shown in figure 1B. Compared with nonactivated platelets, the fractions of PMPs exposing P-selectin or CD63 increased on incubation with TRAP or A23187 (P <0.001 for both P-selectin and CD63, using either agonist). Numbers of PMP released in response to stimulation with TRAP were not different when compared with nonactivated platelets (Fig. 1C).
Only stimulation with A23187 increased PMP numbers (P < 0.001). The baseline characteristics of patients and controls are shown in table 1. There were no significant differences in age and sex between the older healthy persons and the other groups (young healthy persons (except for age) and patients with stable angina pectoris, peripheral arterial disease, NSTEMI, or STEMI). Patients with stable angina had a higher body mass index than the older healthy persons (27.8 vs 23.3 kg/m²; P = 0.012). None of the healthy individuals had clinically manifest cardiovascular disease. In each group studied, the majority of MPs originated from platelets (figure 2A). The median overall fraction of CD61-exposing MPs was 96% (interquartile range, 93%–98%), and the numbers did not differ among groups (P = 0.954, Kruskal–Wallis test). The other MPs (Fig. 2B) originated from erythrocytes (positive for glycophorin A; median, 4%; interquartile range, 1%–7%) and to a lesser extent (≤ 2%) from T-helper cells (CD4), T-suppressor cells (CD8), monocytes (CD14), B cells (CD20), endothelial cells (CD62e), and granulocytes (CD66e). Because no differences among groups were observed regarding numbers and cellular origin of the nonplatelet MPs, the data are summarized for all groups in figure 2B.
Table 1. Baseline characteristics of persons included in the study.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Young healthy subjects</th>
<th>Older healthy subjects</th>
<th>Stable angina</th>
<th>Peripheral arterial disease</th>
<th>NSTEMI</th>
<th>STEMI</th>
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<td>Age (± SD) (years)</td>
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<td>64 (±8)</td>
<td>64 (±10)</td>
<td>68 (±12)</td>
<td>61 (±11)</td>
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<td>9</td>
<td>8</td>
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<td>23.3 (±4.5)</td>
<td>27.8 (±2.4) †</td>
<td>25.3 (±5.0)</td>
<td>27.7 (±5.1)</td>
<td>25.4 (±2.8)</td>
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<td>4 †</td>
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<td>2</td>
<td>6 †</td>
<td>5 †</td>
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<tr>
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<td>7 †</td>
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<td>Beta blockers</td>
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<td>4 †</td>
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<td>5 †</td>
<td>4 †</td>
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<td>2</td>
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</table>

* Documented myocardial infarction or coronary artery disease in parents or siblings before the age of 60; † Daily use of aspirin in previous 7 days; †† p<0.05 compared to older healthy subjects; †† † p<0.001 compared to older healthy subjects.
Figure 2. Plasma microparticle numbers
(A), numbers of CD61-positive MPs in patients and healthy groups; (B), numbers of MPs per cell-specific markers for all groups. Bars represent median (interquartile range, error bars).

Representative dot-plots of CD61-exposing (P)MPs, as detected in an older healthy person (panel A) and a NSTEMI patient (panel B) are shown in figure 3. Staining with anti-P-selectin revealed that the NSTEMI patient had a relatively large subpopulation of PMPs that exposed P-selectin (figure 3D, upper right quadrant) compared with the healthy control (figure 3C). As is also evident in figure 3, all P-selectin-exposing MPs were PMPs, i.e., the upper left quadrants of panels C and D do not show any events.
Figure 3. Origins of P-selectin exposing MPs
(A and B), MPs from a representative healthy person (A) and a representative NSTEMI patient (B) were stained with anti-CD61-FITC. (C and D), triple staining of MPs from a healthy person (C) and a representative NSTEMI patient (D) with annexin V-allophycocyanin, anti-CD61-FITC, and anti-P-selectin-PE shows that P-selectin was exposed exclusively on CD61-positive MPs.

The fractions of PMPs that exposed P-selectin (panel A) or CD63 (panel B) are shown in figure 4. Comparison among groups (Kruskal–Wallis test) showed significant differences for both P-selectin and CD63 (P < 0.001 and P = 0.007, respectively). Compared with the young healthy group, the P-selectin-exposing subpopulations were increased in older individuals (P = 0.028) and were further increased in NSTEMI (P = 0.007) and STEMI (P = 0.045) patients. CD63-exposing subpopulations were increased in patients with peripheral arterial disease (P = 0.041), NSTEMI (P < 0.001), and STEMI (P = 0.049). Previous use of aspirin in NSTEMI (6 of 11 patients) and STEMI patients (4 of 10 patients) did not affect the exposure of P-selectin or CD63 (data not shown). The numbers of PMPs exposing P-selectin or CD63 correlated strongly (r = 0.581; P < 0.001). The concentrations of TAT and F₁⁺₂ are shown in table 2. There were
no significant differences in TAT and F\textsubscript{1,2} concentrations among groups (Kruskal-Wallis test), and there were no correlations between subpopulations of PMPs exposing P-selectin or CD63 and concentrations of TAT \( r = 0.086 \ (P = 0.512) \) and \( r = 0.055 \ (P = 0.677) \), respectively or \( F\textsubscript{1,2} \) \( r = -0.131 \ (P = 0.314) \) and \( r = -0.166 \ (P = 0.209) \), respectively. MP-associated P-selectin constituted only 2.2 (4.7)% [mean (SD)] of total concentrations of sP-selectin in plasma, as assessed from the difference between concentrations of P-selectin in MP-containing plasma and in MP-depleted plasma after high-speed centrifugation (30 min at 17 570g and 20 °C). We found no correlations between the P-selectin-exposing subpopulation of PMPs and either the total concentrations of sP-selectin \( r = 0.153; P = 0.240 \) or the calculated MP-associated fraction of sP-selectin \( r = -0.003; P = 0.991 \).

**Table 2.** Markers of inflammation and coagulation.

<table>
<thead>
<tr>
<th></th>
<th>Young healthy subjects n=10</th>
<th>Older healthy subjects n=10</th>
<th>Stable angina arterial disease n=10</th>
<th>NSTEMI n=11</th>
<th>STEMI n=10</th>
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<tbody>
<tr>
<td>TAT (\mu g/l)</td>
<td>3.28 (2.41-4.68)</td>
<td>3.65 (2.16-4.27)</td>
<td>3.02 (2.34-3.43)</td>
<td>3.82 (3.06-4.84)</td>
<td>3.85 (3.24-5.06)</td>
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<tr>
<td>F\textsubscript{1,2} (nM/l)</td>
<td>0.68 (0.60-0.94)</td>
<td>0.89 (0.80-1.11)</td>
<td>0.85 (0.58-1.12)</td>
<td>0.91 (0.83-1.10)</td>
<td>0.82 (0.66-0.98)</td>
</tr>
</tbody>
</table>

Data represent median values with interquartile ranges in parentheses. * \( P < 0.05 \), Mann-Whitney U-test, for the difference with young healthy controls.

![Graph showing % of P-selectin-exposing PMPs](image-url)
Discussion

The present study shows that subpopulations of PMP exposing P-selectin or CD63 reflect platelet activation in vitro more closely than overall numbers of PMPs. Because platelet activation increases with age and in atherosclerotic disease (21, 22), we studied the presence of PMPs and PMP subpopulations in aging and in atherosclerotic disease in vivo. In our study, the majority of circulating MPs originated from platelets in all individuals. PMP subpopulations, but not the total numbers of PMPs, increased with age and in patients with peripheral arterial disease and in MI.

Gawaz et al. (23) reported that, in the early stage of myocardial infarction the fraction of P-selectin-exposing platelets was increased but not the numbers of PMPs. This early stage was followed after 8 h by an increase in the numbers of PMPs and by a concomitant decrease in platelet counts. These data indicated that the total numbers of PMP are not an early and sensitive marker of platelet activation in vivo. Rather, the late rise of PMP and decrease in platelet count may be a consequence of altered thrombopoiesis in response to the inflammatory reaction during myocardial infarction (24). Thus, discrepancies occur between exposure of P-selectin on platelets and the numbers of PMPs which we have found in vitro and others in vivo (6, 7, 12-
14). These discrepancies may be attributed, at least in part, to the persistent release of MPs by megakaryocytes. Such MPs may be erroneously identified as PMPs, because of similar antigenic profiles (25).

Our results thus indicate that PMP subpopulations may constitute a novel (plasma) marker for in vivo platelet activation. This marker not only reflects platelet activation better than the total numbers of circulating PMPs, but is also more feasible than measuring the exposure of P-selectin on platelets. As the major fraction of sP-selectin in plasma is not associated with MPs, direct determination of the (P)MP-associated fraction of sP-selectin by ELISA is impossible. We are currently developing an ELISA capture assay for both CD61 and P-selectin.

Determination of PMP subpopulations may also be used in monitoring the risk reducing effects of anti-platelet drugs. With regard to the present study, we found ongoing platelet activation despite prior use of aspirin. This is in line with reported increased platelet activation after coronary stent implantation under aspirin use (26). Thus, the risk-reducing properties of aspirin may, at least in part, be related to other mechanisms rather than attenuation of platelet activation.

In conclusion, our in vitro data show that PMP subpopulations reflect the platelet activation status. Increased concentrations of circulating PMP subpopulations are found in ageing, and further increases are encountered in peripheral arterial disease and MI.

We gratefully acknowledge Prof. Dr. D.A. Legemate from the department of Vascular Surgery, AMC, Amsterdam and L.M. Pronk for her technical assistance.

The study was supported by the Netherlands Heart Foundation (Grant 2000B136).
References


