Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events

Aileen M. Healy, PhD; Michael D. Pickard, MA; Aruna D. Pradhan, MD; Yunmei Wang, PhD; Zhiping Chen, MS; Kevin Croce, MD, PhD; Masashi Sakuma, MD; Can Shi, PhD; Alexandre C. Zago, MD; Joseph Garasic, MD; Andrew I. Damokosh, PhD; Tracy L. Dowie, BS; Louis Poisson, MS; James Lillie, PhD; Peter Libby, MD; Paul M Ridker, MD; Daniel I. Simon, MD

Background—Platelets participate in events that immediately precede acute myocardial infarction. Because platelets lack nuclear DNA but retain megakaryocyte-derived mRNAs, the platelet transcriptome provides a novel window on gene expression preceding acute coronary events.

Methods and Results—We profiled platelet mRNA from patients with acute ST-segment–elevation myocardial infarction (STEMI, n=16) or stable coronary artery disease (n=44). The platelet transcriptomes were analyzed and single-gene models constructed to identify candidate genes with differential expression. We validated 1 candidate gene product by performing a prospective, nested case-control study (n=255 case-control pairs) among apparently healthy women to assess the risk of future cardiovascular events (nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death) associated with baseline plasma levels of the candidate protein. Platelets isolated from STEMI and coronary artery disease patients contained 54 differentially expressed transcripts. The strongest discriminators of STEMI in the microarrays were CD69 (odds ratio 6.2, \(P<0.001\)) and myeloid-related protein-14 (MRP-14; odds ratio 3.3, \(P=0.002\)). Plasma levels of MRP-8/14 heterodimer were higher in STEMI patients (17.0 versus 8.0 \(\mu g/mL\), \(P<0.001\)). In the validation study, the risk of a first cardiovascular event increased with each increasing quartile of MRP-8/14 (\(P_{\text{trend}}<0.001\)) such that women with the highest levels had a 3.8-fold increase in risk of any vascular event (\(P<0.001\)). Risks were independent of standard risk factors and C-reactive protein.

Conclusions—The platelet transcriptome reveals quantitative differences between acute and stable coronary artery disease. MRP-14 expression increases before STEMI, and increasing plasma concentrations of MRP-8/14 among healthy individuals predict the risk of future cardiovascular events. (Circulation. 2006;113:2278-2284.)

Key Words: platelets ■ myocardial infarction ■ transcriptional profiling ■ myeloid-related protein-14

Clinical Perspective p 2284

Acute myocardial infarction commonly results from the atherosclerotic plaque disruption\(^1\) and thrombosis that causes coronary arterial occlusion.\(^2\) Angioscopic\(^3\) and pathological\(^1\) observations indicate that platelets constitute a major component of such thrombi, yet the precise molecular events in platelets that immediately precede acute myocardial infarction remain uncertain. Transcriptional profiling can yield mechanistic insights unbiased by preexisting disease hypotheses; however, gene expression after acute myocardial infarction may reflect either triggering events or downstream consequences of plaque rupture and thrombosis, thereby precluding definitive conclusions about causality.

Platelets, generated from complex cytoplasmic extensions from bone marrow megakaryocytes, retain megakaryocyte-derived mRNAs\(^4\) and translational machinery for protein biosynthesis.\(^5\) Human platelets also possess a functional spliceosome, a complex that processes pre-mRNAs into mature message that is translated into protein.\(^6\) Highly purified human platelets contain \(>2000\) transcripts.\(^7\) Because platelets are anuclear, the platelet transcriptome mirrors megakaryocyte-derived mRNAs and represents an averaged mRNA profile of variably aged platelets (platelets circulate

Received December 9, 2005; revision received March 17, 2006; accepted March 20, 2006.

From Millennium Pharmaceuticals, Inc (A.M.H., M.D.P., A.I.D., T.L.D., L.P., J.L.), Cambridge, Mass; Cardiovascular Division (A.D.P., Y.W., Z.C., K.C., M.S., C.S., A.C.Z., P.L., P.M.R., D.I.S.), the Center for Cardiovascular Disease Prevention (A.D.P., P.M.R.), and Donald W. Reynolds Center for Clinical Cardiovascular Research (A.D.P., P.L., P.M.R.), Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass; and Cardiology Division (J.G.), Massachusetts General Hospital, Harvard Medical School, Boston, Mass.

The online-only Data Supplement can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.105.607333/DC1.

Guest Editor for this article was Harvey D. White, DSc.

Correspondence to Daniel I. Simon, MD, Brigham and Women’s Hospital, 75 Francis St, PBB-1, Boston, MA 02115. E-mail dsimon@rics.bwh.harvard.edu

© 2006 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/CIRCULATIONAHA.105.607333
for 7 to 10 days). Thus, transcriptional profiling of platelets provides a novel window on gene expression that precedes acute coronary events, without the confounding possibility that the acute event itself has provoked new gene transcription. We profiled platelet mRNA from patients with acute ST-segment–elevation myocardial infarction (STEMI) or stable coronary artery disease (CAD). The platelet transcriptomes were then analyzed and single-gene models constructed to identify candidate genes with differential expression. We then sought to validate our gene target in a prospective, nested case-control study of incident cardiovascular events among a nationwide population of otherwise healthy individuals without known cardiovascular disease at baseline.

### Methods

#### Patient Selection

Patients undergoing coronary angiography at the Brigham and Women’s Hospital were enrolled in an institutional review board–approved protocol, and all patients provided written consent. Patient groups included those with (1) CAD (history of angina and/or positive noninvasive test and angiographic CAD [ie, stenosis >50%]) and (2) STEMI (patients presenting to the cardiac catheterization laboratory with chest pain [>15 minutes] and ECG ST-segment elevation [>0.5 mm in 2 contiguous leads] for reperfusion with primary angioplasty).

#### Blood Collection and Platelet Isolation

Blood (50 mL) was collected from the arterial sheath before angiography and anticoagulated with PPACK (Hematologic Tech, Essex Junction, Vt). Platelets were isolated within 2 hours by differential centrifugation.8 Purity of platelet preparations was determined by fluorescent-activated cell sorting with phycoerythrin-conjugated mouse anti-CD41a and anti-CD45 antibodies. The final platelet pellet was resuspended in RNA lysis buffer (RNA-STAT-60, Clontech, Mountain View, Calif) for RNA isolation. Expression–polymerase chain reaction (RT-PCR) protocols are included in the Appendix (in the online-only Data Supplement).

#### RNA Extraction, Microarray Hybridization, and Real-Time Polymerase Chain Reaction

RNA isolation, microarray hybridization, and real-time reverse transcription–polymerase chain reaction (RT-PCR) protocols were described previously.9

#### Statistical Methods

Expression data for Affymetrix probe sets (~60 000) were processed with Affymetrix Microarray Suite 5.0 software (Affymetrix, Santa Clara, Calif). The natural log-transformed data were filtered by first eliminating probe sets with <15% present calls and then eliminating probe sets with low variance across all patients.

Standard linear logistic regression analysis was used to find the optimal prediction model for the probability of being a STEMI versus a CAD patient. Clinical variables considered were age, gender, history of diabetes mellitus, cholesterol levels, triglyceride levels, and smoking status. Model selection was accomplished by the score method to find the best-fitting model that excluded all levels, and smoking status. Model selection was accomplished by the score method to find the best-fitting model that excluded all covariates.10

To adjust for multiple comparisons, a permutation test was used which in the disease classifications of the patients were randomly reassigned 2000 times.9 For each permutation, all models were rerun, and the permuted model with the lowest genomic variable probability value was recorded. To obtain the adjusted probability value for a given genomic variable, the fraction of permutations in which the probability value from the observed (true) classification was less than the lowest permuted probability value was calculated. To avoid cases in which permuted data resulted in an unstable logistic regression model, a specialized form of the model was employed.10

Differences in medians were assessed with the Wilcoxon rank sum test. The Fisher exact test was used to assess differences in proportions. All analyses were performed with a statistical software package (SAS Institute).

#### Validation-Phase Study Participants

We tested the validity of findings from the STEMI and CAD patients in a cohort of apparently healthy women 45 years of age or older who were participants in the Women’s Health Study11 and who were followed up prospectively for the occurrence of first cardiovascular event, including nonfatal myocardial infarction, nonfatal stroke, and cardiovascular death. We designed a prospective, nested case-control study in which women who developed a future cardiovascular event (cases, n=255) were matched in a 1:1 ratio with women who remained free of incident cardiovascular disease during follow-up (controls, n=255). Cases and controls were matched with regard to age (within 1 year) and smoking status (former smoker, current smoker, or nonsmoker). When these criteria were applied, 255 matched case-control pairs were available for analysis.

#### Validation-Phase Procedures

Baseline blood samples were collected in tubes that contained EDTA, and plasma samples were stored in liquid nitrogen. Baseline blood samples were collected during the run-in phase, when all participants in the study were given placebo medications. Case and control specimens were assayed for high-sensitivity C-reactive protein (CRP) and lipids, as described previously.12 Plasma myeloid-related protein-8 (MRP-8/14) levels were measured by ELISA (Buhlmann Laboratories, Schonenbuch, Switzerland). Performance characteristics of this assay indicate intra-assay precision of 3.8%, interassay precision of 7.2%, analytical sensitivity of 3.0 ng/mL, and functional sensitivity of 5.6 ng/mL. Samples were handled in a blinded fashion throughout the study.

#### Validation-Phase Statistical Analysis

We used repeated-measures analysis (SAS version 8.01; SAS Institute) to evaluate differences in means between cases and controls with case-control pair modeled as a random effect. Because several variables were skewed, differences in medians were assessed with the signed rank test. The χ2 statistic conditioned on case-control pair was used to assess differences in proportions. Each approach listed allows for correlation within matched case-control sets. Conditional logistic regression was used to estimate relative risk (RR) and 95% confidence interval (CI) after the population was divided into groups based on quartile cutoffs for the control distribution of MRP-8/14. Tests for linear trends were computed with an ordinal variable for biomarker quartiles.

Adjusted risk estimates were obtained from regression models that, in addition to accounting for matching, adjusted for cardiovascular risk factors including history of hypertension, history of diabetes mellitus, body mass index (in kg/m²), parental history of CAD before the age of 60 years, use of hormone replacement therapy, baseline total cholesterol:high-density lipoprotein (TC:HDL) ratio, and CRP. All probability values were 2 tailed, and values of <.05 were considered to indicate statistical significance.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

#### Results

Microarray Analyses of Platelet Samples

We profiled platelet mRNA from patients presenting to the cardiac catheterization laboratory with chest pain and ST-segment elevation for reperfusion with primary angioplasty.
TABLE 1. Baseline Characteristics of Patients in the CAD and STEMI Cohorts

<table>
<thead>
<tr>
<th></th>
<th>CAD (n=44)</th>
<th>STEMI (n=16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean±SD, y</td>
<td>63.7±10.6</td>
<td>55.3±15.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Smoking status, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>13.6</td>
<td>62.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Past</td>
<td>22.7</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>63.6</td>
<td>31.3</td>
<td></td>
</tr>
<tr>
<td>Male, %</td>
<td>72.7</td>
<td>75.0</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>63.6</td>
<td>37.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>34.1</td>
<td>12.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>56.8</td>
<td>31.3</td>
<td>0.14</td>
</tr>
<tr>
<td>Use of aspirin, %</td>
<td>72.7</td>
<td>50.0</td>
<td>0.13</td>
</tr>
<tr>
<td>White blood cell count, 10^9/µL (IQR)</td>
<td>7.2 (6.2–8.6)</td>
<td>10.3 (8.1–14.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>Platelet count, mean±SD, 10^9/µL</td>
<td>237±55</td>
<td>234±48</td>
<td>0.82</td>
</tr>
<tr>
<td>No. of diseased vessels, mean±SD</td>
<td>1.84±1.0</td>
<td>1.81±0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>No. of diseased vessels, %</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13.6</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20.5</td>
<td>50.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>34.1</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>31.8</td>
<td>31.3</td>
<td></td>
</tr>
</tbody>
</table>

*P values derived from the t test and Wilcoxon rank-sum test for normally and nonnormally distributed continuous variables, respectively, and the Fisher exact test for categorical covariates.

(n=16) or stable CAD (n=44). Patients with stable CAD were selected as the comparator group rather than normal subjects to focus on the differential expression of genes associated with plaque rupture and thrombosis. Baseline characteristics of study patients are shown in Table 1. STEMI patients were younger, reported more tobacco use, and presented with apparently fewer cardiac risk factors than CAD patients; however, only smoking status reached statistical significance. The extent of angiographic CAD was similar between the 2 groups. Platelets isolated from STEMI and CAD patients contained transcripts represented by >18 000 probe sets. We selected the 1000 probe sets with the highest variance for analysis. When we used logistic regression and adjusted for smoking status, 54 probe sets had estimates with P<0.05, before adjustment for multiple comparisons. Of the 54 probe sets, 29 showed increased and 25 showed decreased expression in STEMI compared with CAD (for a complete list, see the Appendix, supplemental Tables I and II, in the online Data Supplement). CD69 was the most significant probe set showing increased expression in STEMI, with 2.7-fold higher median expression in STEMI than in CAD, followed by MRP-14, which had 2.2-fold higher median expression in STEMI (Table 2, shown as log-transformed expression intensity). After adjustment for smoking status, the OR for having a STEMI event was 6.2 (95% CI 2.3 to 29.8) per 1-log increase in CD69 expression intensity and 3.3 (95% CI 1.5 to 9.0) for MRP-14 (Table 2).

CD69 and MRP-14 RNA Levels in Platelets and Megakaryocytes by RT-PCR

CD69 and MRP-14 are of particular interest in generating disease hypotheses. CD69 is widely expressed in hematopoietic cells,13 and engagement of CD69 on platelets results in thromboxane production and aggregation.14 MRP-14 (S100A9, calgranulin B) is a member of the S100-family of Ca2+-modulated proteins that have intracellular and extracellular roles modulating calcium signaling, arachidonic acid metabolism, cytoskeletal reorganization, and leukocyte trafficking.15

We confirmed the presence of CD69 and MRP-14 expression in platelets by real-time RT-PCR. CD69 levels were 3.7-fold (P<0.01) higher and MRP-14 levels were 2.2-fold (P<0.001) higher in STEMI patients. To demonstrate that both transcripts were platelet derived, we examined bone marrow megakaryocytes for expression of CD69 and MRP-14 transcripts. Freshly isolated human bone marrow megakaryocytes and megakaryocytes generated in vitro by differentiation of human CD34-positive cells expressed high levels of both transcripts (see Appendix, supplemental Figure I, in the online Data Supplement for MRP-14 expression data).

Platelet preparations contained minimal leukocytes: 0.28% (interquartile range [IQR] 0.03% to 0.60%) CD45-positive cells in STEMI and 0.33% (IQR 0.00% to 2.63%) in CAD samples. Real-time RT-PCR for leukocyte markers, including CD3, CD14, CD19, and FUT4 (fucosyltransferase 4), yielded signals that approached background levels. CD69 expression levels were 1.02-fold higher (P=0.97) in STEMI patients, whereas FUT4 levels were 1.04-fold higher (P=0.91) in CAD patients. These observations indicate that MRP-14 and CD69 transcripts originate from platelets and suggest that the increased expression of CD69 and MRP-14 detected in STEMI patient samples is likely platelet derived.

TABLE 2. CD69 and MRP-14 Microarray Results

<table>
<thead>
<tr>
<th></th>
<th>STEMI (n=16)</th>
<th>CAD (n=44)</th>
<th>P</th>
<th>STEMI:CAD, OR (95% CI)</th>
<th>Adjusted P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD69</td>
<td>5.9 (5.1, 6.3)</td>
<td>4.9 (4.5–5.3)</td>
<td>0.002</td>
<td>6.2 (2.3–29.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRP-14</td>
<td>6.2 (6.0, 6.8)</td>
<td>5.4 (4.8–5.9)</td>
<td>0.001</td>
<td>3.3 (1.5–9.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*P values derived from Wilcoxon rank-sum test or Fisher exact test (see Methods).
†P value adjusted for multiple comparisons with permutation test.
STEMI Patients Have Elevated Plasma Levels of MRP-8/14

The increased expression of MRP-14 in STEMI patients at the mRNA level led us to quantify plasma protein levels of the most abundant form of MRP-14, the MRP-8/14 (S100A8/H11005), in these same patients. We found a significant increase in plasma levels of MRP-8/14 in STEMI (median 17 μg/mL, IQR 14.8 to 19.7) compared with CAD (8.0 μg/mL, IQR 5.0 to 10.6; P<0.001). CRP levels trended higher in STEMI (median 2.9 to 15.5) than in CAD (3.6 mg/L, IQR 2.6 to 5.4) patients, but this did not reach statistical significance (P=0.24). There were no significant differences in total cholesterol (P=0.81) or triglyceride levels (P=0.97). CD69 was not pursued further in the present study because it is a membrane protein without secreted forms, and analysis of protein levels would require a flow-cytometry–based assay of freshly isolated platelets.

Validation in a Primary Prevention Population

A substantial proportion of cardiovascular events are sudden death or first myocardial infarction or stroke without antecedent symptoms. For this reason, we sought to validate the MRP-14 gene target by performing a prospective, nested case-control study (n=255 case-control pairs) among 28,345 apparently healthy postmenopausal women followed up in the Women’s Health Study11 to assess the risk of future cardiovascular events (nonfatal myocardial infarction or stroke, or cardiovascular death) associated with baseline plasma levels of MRP-8/14 with a median follow-up time of 2.9 years (range 0.01 to 5.9 years). We performed the validation study in apparently healthy individuals to avoid evaluation of patients presenting with acute coronary syndromes (ACS), which are accompanied by local and systemic inflammation and myocardial necrosis that would likely influence MRP-8/14 levels.

Baseline characteristics of study participants are shown in Table 3. As anticipated, cases (111 myocardial infarctions, 111 strokes, and 33 cardiovascular deaths) had higher rates of cardiovascular risk factors than controls. Because of matching, age and smoking status were virtually identical between study groups.

Initially healthy women who subsequently developed cardiovascular events during follow-up (cases) had higher median MRP-8/14 levels at baseline than women who remained free of disease (controls; P<0.001). Spearman correlation coefficients between MRP-8/14 and TC, low-density lipoprotein (LDL), HDL, TC:HDL, and CRP were 0.16, 0.20, −0.35, 0.41, and 0.40 (all P<0.01). Table 4 presents the crude and multivariable-adjusted RR of a first cardiovascular event according to increasing quartiles of baseline MRP-8/14. In matched-pair analysis that accounted for age and smoking status, the RR increased significantly with each increasing quartile of baseline concentration of MRP-8/14 (PRim <0.001), such that the women in the highest versus lowest quartile had a 4-fold elevation in risk (RR 3.8, P<0.001). After adjustment for traditional cardiovascular risk factors (history of hypertension, history of diabetes mellitus, body mass index, parental history of CAD before the age of 60 years, use of hormone replacement therapy, and baseline TC:HDL), the RRs according to increasing quartiles of MRP-8/14 were 1.0, 1.7, 1.7, and 2.3 (PRim =0.033). When additionally adjusted for CRP, a risk gradient persisted (PRim =0.047), with women in the highest quartile having a 2.3-fold increase in risk of any vascular event (95% CI 1.1 to 4.7, P=0.03). Analyses that

### Table 3. Baseline Characteristics of Participants in the Prospective Nested Case-Control Validation Study

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=255)</th>
<th>Cases (n=255)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>White race, %</td>
<td>97.7</td>
<td>94.5</td>
<td>0.07</td>
</tr>
<tr>
<td>Age, mean±SD, y</td>
<td>61.0±8.7</td>
<td>61.0±8.7</td>
<td>...</td>
</tr>
<tr>
<td>Smoking status, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>22.4</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>Past</td>
<td>35.7</td>
<td>35.7</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>42.0</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td>Body mass index, mean±SD, kg/m²</td>
<td>25.5±4.4</td>
<td>27.1±5.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>31.0</td>
<td>55.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parental history of CAD, %</td>
<td>11.7</td>
<td>15.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>2.0</td>
<td>12.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>36.9</td>
<td>46.3</td>
<td>0.03</td>
</tr>
<tr>
<td>MRP-8/14, μg/mL</td>
<td>2.1 (1.2–3.4)</td>
<td>2.7 (1.6–4.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC, mg/dL</td>
<td>213.0 (191.0–240.0)</td>
<td>226.0 (196.0–254.0)</td>
<td>0.006</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>126.4 (104.3–147.0)</td>
<td>134.5 (110.0–156.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>51.5 (41.7–63.1)</td>
<td>45.8 (38.0–56.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>127.0 (87.0–187.0)</td>
<td>166.0 (111.0–256.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>2.3 (0.9–4.0)</td>
<td>3.2 (1.6–6.1)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
evaluated cases of myocardial infarction separately (n=111) yielded similar results. When matched for age and smoking status, women in the highest quartile had a risk of future myocardial infarction that was almost 7 times that of the lowest quartile (RR 6.9, 95% CI 2.3 to 20.9, \(P=0.001\)). The multivariable-adjusted RRs across quartiles of MRP-8/14 were 1.0, 2.1, 3.0, and 3.3, respectively (\(P_{\text{trend}}=0.08\)).

To illustrate the potential ability of MRP-8/14 to add prognostic value to lipid- or CRP-based screening, we computed the RR of cardiovascular events after study participants were stratified into 9 groups according to tertiles of MRP-8/14 and tertiles of total cholesterol:HDL or CRP. Women with low TC:HDL or CRP and low levels of MRP-8/14 had the lowest RR (Figure). In contrast, women with high TC:HDL or CRP and high levels of MRP-8/14 had the highest RR. Importantly, even among women with low or intermediate TC:HDL or CRP levels, the risk of cardiovascular events was greater among those with high than with lower levels of MRP-8/14.

### Discussion

The present study demonstrates increased expression of platelet CD69 and MRP-14 in patients presenting to the cardiac catheterization laboratory with STEMI and further documents in a prospective, nested case-control study that the baseline plasma concentration of MRP-8/14 predicts the risk of a first cardiovascular event in apparently healthy women. Elevated serum levels of MRP-8/14 are a useful biomarker of disease activity in inflammatory disorders, such as rheumatoid arthritis and Crohn disease.\(^1\) Neutrophils and monocytes highly express MRP-8/14.\(^1\) Inflammatory stimuli promote the surface expression and secretion of MRP-8/14, where it functions, in part, as a chemoattractant that regulates leukocyte adhesion.\(^1\) Patients with diabetes mellitus have elevated plasma levels of MRP-8/14,\(^2\) and related S100/calgranulin family members bind to RAGE (receptor for advanced-glycation end products) and trigger proinflammatory and prothrombotic responses.\(^2\) In addition to MRP-14, MRP-8 was also detected in platelets by microarray and

### Table 4: RRs for Incident Cardiovascular Events According to MRP-8/14 Levels

<table>
<thead>
<tr>
<th>Quartile of MRP-8/14 (Range)</th>
<th>Controls, n (%)</th>
<th>Cases, n (%)</th>
<th>RR (95% CI)</th>
<th>(P_{\text{trend}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.15 µg/mL</td>
<td>64 (25.1)</td>
<td>30 (11.8)</td>
<td>1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1.16–2.05 µg/mL</td>
<td>64 (25.1)</td>
<td>59 (23.1)</td>
<td>2.1</td>
<td>0.001</td>
</tr>
<tr>
<td>2.06–3.35 µg/mL</td>
<td>64 (24.1)</td>
<td>65 (25.5)</td>
<td>3.0</td>
<td>0.01</td>
</tr>
<tr>
<td>&gt;3.36 µg/mL</td>
<td>63 (24.7)</td>
<td>101 (39.6)</td>
<td>3.3</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Matched pairs analysis: matched on age and smoking status. Risk factor–adjusted: adjusted for body mass index (kg/m\(^2\)), history of diabetes mellitus, history of hypertension, parental history of premature CAD, TC:HDL, and use of hormone replacement therapy.
RT-PCR analyses (data not shown). Although plasma MRP-8/14 was previously considered leukocyte derived,\(^1\) the present study suggests that platelets and megakaryocytes may serve as an additional source of MRP-8/14. Because of its ability to regulate calcium signaling and promote cytoskeletal reorganization,\(^2\) it is intriguing to speculate that MRP-14 may be involved in the cellular events that promote platelet-mediated thrombosis. Preliminary data from our laboratory (Y.W., Z.C., K.C., M.S., D.I.S.) indicate that MRP-8/14 protein is present in human platelets as determined by flow cytometric analysis and that monoclonal antibody blockade of MRP-14 inhibits shear-induced thrombus formation. The present study design does not permit us to determine the precise cellular source of plasma MRP-8/14. The relative contributions of platelets, leukocytes, and endothelial cells to the plasma pool of MRP-8/14 are the focus of ongoing studies.

The present results have several important implications. First, the data suggest that MRP-8/14 can independently predict risk of future cardiovascular events and may add prognostic information to that conveyed by standard risk factors and CRP. Second, the findings support evidence linking inflammation and thrombosis in the pathogenesis of ACS.\(^3\) Third, the results of the present study implicate the platelet transcriptome as a possible source of bioactive molecules or markers for disease. The precise mechanisms that result in measurable differences between STEMI and CAD platelets remain unclear. Patients with ACS have altered platelet reactivity.\(^4\) Differential RNA levels in the platelets of ACS patients may reflect differences in platelet function. Circulating platelets not only respond to extracellular signals by upregulating expression of preformed molecules but also engage in new protein synthesis.\(^5\) Alternatively, megakaryocytes may respond to soluble signals unique to the ACS patient. The RNA profile of circulating platelets reflects such changes in bone marrow megakaryocytes. Differences in platelet RNA levels may also result from changes in the circulating pool of platelets, including platelet age and shortening of platelet half-life, or from alterations in platelet mRNA stability. Further study is required to elucidate these differences.

Several limitations of the study merit consideration. First, we cannot rule out a contribution by leukocytes to our profiling data. Nonetheless, we verified that leukocyte contamination was lower in STEMI patients and that the platelet preparations did not contain neutrophil and monocyte markers. We detected MRP-14 transcripts in megakaryocytes, and the presence of MRP-14 mRNA in platelets has been reported previously with filtration procedures used to minimize leukocyte contamination.\(^6\) Second, whether or not aspirin impacts levels of MRP-8/14 is unknown; however, baseline blood specimens in the Women’s Health Study were collected during a 3-month run-in phase when all participants in the study were taking placebo medications, which thereby eliminated any potential influence aspirin might have had on baseline MRP-8/14 levels. Furthermore, adjustment for subsequent randomized assignment to aspirin did not affect our results. Third, levels of MRP-8/14 may be increased in association with smoking. Among control subjects included in the nested case-control study, MRP-8/14 levels were 2.3 and 2.0 \(\mu\)g/mL in current smokers and never-smokers, respectively (\(P=0.09\)). However, it is important to note that both the profiling and validation studies accounted for smoking status, and furthermore, our results in the nested case-control study did not differ after the exclusion of smokers from the risk-prediction models. The multivariable-adjusted RRs across quartiles of MRP-8/14 were 1.0, 1.2, 1.5, and 2.2 (\(P_{\text{trend}}=0.06\)). Fourth, the clinical phenotypes of the platelet profiling (STEMI) and validation (cardiovascular death, non-fatal myocardial infarction, and stroke) studies differed, which raises the possibility that the diverse causes of cardiovascular death and stroke may have influenced the present analyses. However, when cases of myocardial infarction (\(n=111\)) were analyzed separately, baseline plasma concentration of MRP-8/14 also predicted the risk of first myocardial infarction (RR 6.9, 95% CI 2.3 to 20.9, \(P=0.001\)). Fifth, the relationship between MRP-8/14 and burden of atherosclerotic disease is unknown. More sensitive measures of disease burden (eg, intravascular ultrasound) will be required in future studies. Finally, although CD69 was the most significant probe set that showed increased expression in STEMI, we were unable to validate CD69 in the Women’s Health Study because it is a membrane protein without secreted forms, and analysis of protein levels will require a flow-cytometry–based assay of freshly isolated platelets. This line of investigation is likely to be fruitful, because engagement of CD69 on platelets results in thromboxane production and platelet aggregation.\(^4\)

In conclusion, the platelet transcriptome reveals quantitative differences between acute and stable CAD. Increases in MRP-14 expression before STEMI and increasing plasma concentrations of MRP-8/14 among healthy individuals predict the risk of future cardiovascular events. Further characterization of the platelet transcriptome and proteome, especially analyses of the 54 differentially expressed platelet transcripts, either alone or in combination, will likely increase our understanding of platelet functions in health and disease.

Acknowledgments

This work was supported in part by grants from the National Institutes of Health (HL57506 and HL60942 to Dr Simon) and Millennium Pharmaceuticals. Drs Ridker, Libby, and Pradhan are supported by research funds from the Leducq Foundation (Paris, France), the Doris Duke Foundation (New York, NY), and the Donald W. Reynolds Foundation (Las Vegas, Nev). The authors acknowledge the technical expertise of Katherine Knapp, Angela Quinn, and Russell Walker and the Millennium TRACE group for supporting the RNA expression data and would like to thank Denise Aucoin Barry in her role as research study coordinator.

Disclosures

This study was funded in part by Millennium Pharmaceuticals. Dr Healy, M.D. Pickard, Dr Damokosh, T.L. Dowie, L. Poisson, and Dr Lillie are employees of Millennium Pharmaceuticals and played roles in the design of the study, data collection, data analysis, data interpretation, and the writing of the report. Drs Simon and Libby reported having served as consultants and advisors to Millennium Pharmaceuticals. Dr Ridker is named as a coinventor on patents filed by Brigham and Women’s Hospital that relate to the use of inflammatory biological markers in cardiovascular disease.
REFERENCES


Platelet Expression Profiling and Clinical Validation of Myeloid-Related Protein-14 as a Novel Determinant of Cardiovascular Events

Circulation. 2006;113:2278-2284; originally published online May 8, 2006;
doi: 10.1161/CIRCULATIONAHA.105.607333
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/113/19/2278

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2006/05/08/CIRCULATIONAHA.105.607333.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/