Role of Reticulated Platelets and Platelet Size Heterogeneity on Platelet Activity After Dual Antiplatelet Therapy With Aspirin and Clopidogrel in Patients With Stable Coronary Artery Disease

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Objectives
The aim of this study was to evaluate the relationship between reticulated platelets (RPs), platelet size, and platelet function in patients with stable coronary artery disease (CAD) taking aspirin and clopidogrel.

Background
Reticulated platelets are young platelets that are larger and possibly more active than non-RPs.

Methods
Flow cytometry was used to measure RPs after staining with thiazole orange and to define the upper 20% and lower 20% of platelets by size. Platelet aggregation was measured with light transmission aggregometry (LTA); platelet activation was assessed by measuring activated platelet surface expression of P-selectin and glycoprotein (GP) IIb/IIIa.

Results
Ninety patients were recruited and stratified into tertiles of %RPs. Patients in the upper tertile displayed greater platelet aggregation to 5-μmol/l adenosine diphosphate (ADP) (50.7±16.4% vs. 34.2±17.3%, p < 0.001), 1.5-mmol/l arachidonic acid (AA) (27.3±16.9% vs. 11.7±9.3%, p < 0.001), and 1-μg/ml collagen (18±11.6% vs. 12.1±8.7%, p < 0.05) and greater expression of GP IIb/IIIa (4.7±1.8% vs. 3.1±2.2%, p < 0.001). Frequency of low response to aspirin (AA LTA >20%) was higher in the upper tertile (53% vs. 17%, p < 0.001) compared with the lower tertile; low response to clopidogrel (ADP LTA >50%) was also elevated in the upper tertile (50% vs. 13%, p < 0.003). The larger platelet gate had a higher % of RPs compared with the smaller gate (15.4±16.7% vs. 1.7±2.3%, p < 0.001) and greater GP IIb/IIIa (5.7±3.1 vs. 2.1±1.2, p < 0.001) and P-selectin expression (7.8±4.9 vs. 4.6±2.7, p < 0.001).

Conclusions
The proportion of circulating RPs strongly correlates with response to antiplatelet therapy in patients with stable CAD. Large platelets exhibit increased reactivity despite dual antiplatelet therapy, compared with smaller platelets. (J Am Coll Cardiol 2008;52:743–9) © 2008 by the American College of Cardiology Foundation

Antiplatelet therapies with aspirin and clopidogrel have been widely used to prevent thromboembolic events in selected patients with coronary atherosclerosis (1–5). It is generally accepted that the principal mechanism of this effect occurs through inhibition of platelet activation and aggregation. Several studies have suggested wide interindividual variability in the responses to aspirin and clopidogrel, as assessed with ex vivo platelet function assays (6–8). A low degree of inhibition in the platelets of patients treated with these drugs has been termed “resistance” to aspirin or clopidogrel and has been implicated in adverse cardiovascular outcomes, particularly after percutaneous coronary intervention (9,10). The mechanisms for this phenomenon are unclear, and methods to identify and treat it are still being elucidated.

One possible mechanism is an elevated turnover of the circulating platelet pool, yielding a larger population of young platelets that are more reactive than an older population of platelets. Circulating reticulated platelets (RPs) are identifiable on the basis of staining for messenger ribonucleic acid. This population is generally believed to represent younger platelets, with increased mean volume and a greater number of dense granules than older circulating platelets (11). The RPs are elevated in patients with acute coronary syndromes and stroke (12,13), although it is not clear...
whether this phenomenon is a cause of clinical instability or is a consequence of the syndrome. We recently reported in a population of healthy subjects that an elevated platelet turnover, as reported by the RP fraction, is associated with increased platelet aggregation and activity and decreased responses after a single dose of aspirin (14). It is not known whether platelet responses to chronic aspirin and clopidogrel therapy in patients with coronary artery disease (CAD) are dependent on the proportion of circulating RPs.

Younger platelets tend to be larger in size, and thus large platelets could be a reflection of the number of RPs, although not all large platelets are young platelets. It is also believed that platelet size is determined at the time of megakaryocyte production and release of platelets (15). Platelets with increased platelet volume are more reactive hemostatically, aggregate more rapidly in response to collagen (16), synthesize more thromboxane B2 (17,18), release more serotonin granules, and express more glycoprotein (GP) Ib and GP IIb/IIIa (19). It is thus possible that large platelets might respond differently to antiplatelet therapy compared with smaller platelets. Large platelets could also potentially affect responses to antiplatelet therapy independent of %RPs, by expressing larger amounts of cyclooxygenase (COX)-1, adenosine diphosphate (ADP) receptors, and alpha-granule proteins, thus making them more reactive. Mean platelet volume (MPV) is a measure of platelet size, is elevated in unstable angina and acute myocardial infarction (MI) (20,21), and is associated with worse cardiovascular outcomes after MI (22) or stroke (23). Whether platelet size, in addition to the presence of younger platelets, plays a role in modifying responses to antiplatelet therapy is not known.

We hypothesized that increased proportions of RPs and platelet size are associated with increased platelet reactivity despite dual antiplatelet therapy in patients with stable CAD taking both aspirin and clopidogrel. We sought to primarily use flow cytometry platelet size data along with MPV values to evaluate platelet size responses.

**Methods**

**Study population.** The study was approved by the Institutional Review Board of the Methodist Research Institute; all subjects provided informed consent. We enrolled patients with known stable CAD who were admitted to the Methodist DeBakey Heart Center from October 2005 to July 2007, were scheduled to undergo elective cardiac catheterization and/or planned percutaneous coronary inter-vention, and who were taking both clopidogrel (75 mg daily) and aspirin (81 to 325 mg daily) for at least 1 week; no additional loading dose of clopidogrel was administered. These subjects were not recruited consecutively but randomly, and all patients we approached agreed to participate in the study. Exclusion criteria were administration of any GP IIb/IIIa antagonist during the week before the study, acute MI in the week before the study, hemodynamic instability, recent history of cardiac arrest or cardiopulmonary resuscitation, thrombocytopenia (<100 × 10^9 cells/mm^3), anemia (hemoglobin <10 g/dl), or renal failure (creatinine >2.5 mg/dl). Blood samples were collected in tubes containing citrate. The tubes were filled to capacity and then gently mixed. Blood samples were processed within 2 h of blood collection without stasis (by placing in a machine that gently rolls the tubes continuously).

**Light transmission aggregometry.** Turbidimetric platelet aggregation was performed in platelet-rich plasma with a platelet count adjusted to 250 × 10^3/μl. Platelets were stimulated with 5 μmol/l ADP, 1.5 mmol/l arachidonic acid (AA), and 1 μg/ml collagen. Aggregation was performed with a BioData PAP-8 platelet aggregometer (BioData Corp., Horsham, Pennsylvania). The extent of aggregation was defined as the maximal amount of light transmission reached at 6 min after addition of the agonist, with platelet-poor plasma used as a reference. “Low response” to aspirin was defined as platelet aggregation ≥20% in response to 1.5 mmol/l AA. “Low response” to clopidogrel was defined as platelet aggregation ≥50% in response to 5 μmol/l ADP. Both of these definitions have been used in prior reports, and these thresholds of post-treatment platelet aggregation were demonstrated to be associated with a higher risk of ischemic events (10,24–26). “Low response” to both aspirin and clopidogrel was present when patients met both criteria.

**GP IIb/IIIa and P-selectin expression.** Platelet activation was determined by platelet surface expression of P-selectin and GP IIb/IIIa with whole blood flow cytometry as previously described (27). Briefly, activated GP IIb/IIIa was assessed with a fluorescein isothiocyanate (FITC)-conjugated PAC-1 antibody (Becton Dickinson, San Jose, California), and P-selectin expression was determined with an R-phycocerythrin (PE)-conjugated anti-CD62P antibody (BD Pharmingen, San Jose, California). Five microliters of citrated whole blood were diluted with 70 μl of Tyrodes/bovine serum albumin followed by 5 μl of 200-μmol/l ADP (10 μmol/l final concentration) and then by 20 μl of anti-CD62b or PAC-1 antibody directed against integrin.
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Table 1 Demographic and Hematological Characteristics of the Patients in the 3 Tertiles of RPs

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Lower Tertile (n = 30)</th>
<th>Middle Tertile (n = 30)</th>
<th>Upper Tertile (n = 30)</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>67 ± 14</td>
<td>62 ± 10</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>80</td>
<td>77</td>
<td>63</td>
</tr>
<tr>
<td>Race (% Caucasian)</td>
<td>43</td>
<td>53</td>
<td>43</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>34 ± 12</td>
<td>30 ± 5</td>
<td>30 ± 5</td>
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<td>Systolic BP (mm Hg)</td>
<td>127 ± 26</td>
<td>143 ± 21</td>
<td>158 ± 16</td>
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<td>Diastolic BP (mm Hg)</td>
<td>71 ± 17</td>
<td>75 ± 11</td>
<td>75 ± 11</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>73 ± 9</td>
<td>68 ± 12</td>
<td>74 ± 11</td>
</tr>
<tr>
<td>White blood cells (10³/mm³)</td>
<td>7.4 ± 2.2</td>
<td>7.1 ± 2.1</td>
<td>7.6 ± 2</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>13.2 ± 1.5</td>
<td>13.3 ± 1.8</td>
<td>12.7 ± 1.8</td>
</tr>
<tr>
<td>Platelets (10³/mm³)</td>
<td>217 ± 53</td>
<td>199 ± 48</td>
<td>207 ± 60</td>
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<td>MPV (fl)*</td>
<td>9.9 ± 0.6</td>
<td>9.9 ± 0.5</td>
<td>10.5 ± 0.9</td>
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<td>RPs (%)</td>
<td>1.3 ± 0.7</td>
<td>3.1 ± 0.8</td>
<td>17.9 ± 8.1</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>1.3 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>1.4 ± 1.2</td>
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<td>Diabetes (%)</td>
<td>33</td>
<td>27</td>
<td>40</td>
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<tr>
<td>Hypertension (%)</td>
<td>57</td>
<td>57</td>
<td>63</td>
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<tr>
<td>Hyperlipidemia (%)</td>
<td>43</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>23</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Stroke (%)</td>
<td>23</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>CHF (%)</td>
<td>2.2</td>
<td>7.1</td>
<td>10.5 ± 0.9</td>
</tr>
<tr>
<td>History of MI (%)</td>
<td>2.2</td>
<td>7.1</td>
<td>10.5 ± 0.9</td>
</tr>
</tbody>
</table>

*p < 0.05.

α2β3. After incubating for 20 min, the mixture was fixed with phosphate-buffered saline containing 1% paraformaldehyde. Samples were analyzed with a Coulter Epics XL MCL flow cytometer (Beckman-Coulter, Miami, Florida). Unstimulated samples served as negative controls. Both PAC-1 binding and P-selectin expression were expressed as absolute difference in log mean fluorescence intensity between unstimulated and stimulated samples.

RPs. Reticulated platelets were measured with a previously described flow cytometry assay (13,27,28), which is an appropriate method of measuring young platelets and platelet turnover that has been validated in multiple studies (29,30). Five microliters of whole blood were added to a tube containing 1 ml of 10% thiazole orange (ReticCount, Becton Dickinson) and a second tube containing 1 ml of Isoflo (Beckman-Coulter) as a control. After incubation for 30 min in the dark, the tubes were spun at 1,200 g for 2.5 min to form a cellular pellet. The supernatant was discarded, and the pellet was resuspended in 1 ml of Isoflo. Within 1 h, flow cytometry was performed counting 5,000 stained platelets in the platelet gate. The platelets demonstrating an increase in the mean fluorescence intensity beyond a threshold margin set at <1% of all platelets at baseline were counted as RPs and expressed as a percentage of total platelets counted.

Platelet size. Platelet size was obtained with 2 methods: 1) flow cytometry size-gating with forward scatter; and 2) MPV values. With flow cytometry, platelet size gates were established with forward scatter profiles to determine the largest 20% of platelets and the smallest 20% of platelets, to obtain discretely different platelet size populations. This method, however, did not provide absolute platelet sizes. P-selectin expression, PAC-1 antibody binding, and the number of RPs were measured in platelets falling within these gates. The MPV values were obtained with an automated machine along with the hemogram.

Statistical analysis. Patients were stratified into ascending tertiles according to the proportion of circulating RPs. An SD of 10% was assumed, and calculations were based on a power of 80% and a significance level of 0.05. On the basis of the prior study (14), we determined that a sample population of more than 60 patients would be sufficient to demonstrate differences in platelet function with %RPs. Flow cytometry platelet size-gating was used to study the effects of platelet size on platelet function. When using MPV for platelet size, this sample size would have insufficient power to study the platelet effects of MPV (because of the narrow range of MPVs) and hence was not the primary end point of this study. Normally distributed data are expressed as mean ± SD. The Student unpaired t test was used to compare means between any 2 groups. One-way analysis of variance was used to analyze differences in means between tertiles, because data were normally distributed. The chi-square test was used to compare the upper tertile with the other 2 tertiles for dichotomous variables. Multiple linear and logistic regression analysis was performed to test the significance of %RPs and MPV on platelet activation and response rates. A p value ≤0.05 was considered to be statistically significant.

Results

Demographic and hematological characteristics. Clinical and hematological parameters were no different between the tertiles of patients apart from an elevated MPV in the upper tertile (expected because of stratification on the basis of RPs, which are larger). Although the prevalence of congestive heart failure (CHF) was higher in the lower tertile, the actual number of patients with CHF was very low (6 of 30...
in the lower tertile vs. 1 of 30 in the other 2 tertiles) (Table 1). Of 90 patients, 55 were taking 325 mg of aspirin daily and 35 patients were taking 81 mg of aspirin daily before enrollment.

**Platelet aggregation.** Light transmission aggregometry (LTA) in response to 5-μmol/l ADP, 1.5-mmol/l AA, or 1-μg/ml collagen was significantly higher in the upper tertile of RPs compared with both the middle and lower tertiles (Fig. 1, Table 2).

**Platelet activation.** Activated GP IIb/IIIa expression was greater in the upper tertile of patients (4.7 ± 1.8) compared with the middle and lower tertiles (2.9 ± 1.6 and 3.1 ± 2.2, respectively, p < 0.001). However, expression of P-selectin was no different between the 3 tertiles (Table 2).

**Aspirin dose and platelet responses.** There were no differences in either platelet activation or aggregation with either the 81- or 325-mg dose of aspirin. When analyzed in
tertiles of RPs, there were again no differences in platelet function on the basis of the dose (Table 2).

Low response to aspirin and clopidogrel. The frequency of low response to aspirin was significantly higher in the upper tertile (16 of 30, 53%) compared with the middle (3 of 30, 10%) and lower (5 of 30, 17%) tertiles (p < 0.001). Additionally, the frequency of low response to clopidogrel was higher in the upper tertile (15 of 30, 50%) compared with the other 2 tertiles (20% [6 of 30] and 13% [4 of 30] in the middle and lower tertiles, respectively, p = 0.003). The number of patients meeting criteria for both aspirin and clopidogrel low response was also higher in the upper tertile (10 of 30, 33%) compared with the lower (2 of 30, 7%) and middle (0%) tertiles (p < 0.001) (Fig. 2). Compared with the lower 2 tertiles, being in the upper tertile conferred a sensitivity of 83%, specificity of 74%, positive predictive value of 33%, and a negative predictive value of 97% (p < 0.001) of having a low response to both aspirin and clopidogrel.

Influence of platelet size (with flow cytometry) on platelet activation. We observed a greater percentage of RPs in the pool of large platelets (upper 20%) (15.4 ± 16.7%) compared with the pool of small platelets (lower 20%) (1.73 ± 2.3%, p < 0.001) (Fig. 3). Of all RPs, 61% were present in the large pool compared with 7% of all RPs present in the small pool. Greater GP IIb/IIIa expression (5.7 ± 3.1 vs. 2.1 ± 1.2, p < 0.001) as well as higher P-selectin expression (7.8 ± 4.9 vs. 4.6 ± 2.7, p < 0.001) were found in the large compared with the smaller platelets (Fig. 4). Background activation was no different between the large and small platelets.

When stratified by platelet size and aspirin response, large platelets of aspirin low responders had almost twice the number of RPs compared with normal responders (23.5 ± 15.8% vs. 12.5 ± 16.3%, p < 0.05). Expression of GP IIb/IIIa was also elevated in large platelets of aspirin low responders compared with normal responders, whereas expression of P-selectin was no different between the 2 groups (Table 3). Similar platelet activation patterns were seen when stratified by responses to clopidogrel (Table 4). These data suggest that large platelets demonstrate elevated activity, particularly in low responders to antiplatelet therapy, possibly due to an increased proportion of RPs.

Regression analysis. Multiple logistic regression was performed with age, gender, aspirin dose, MPV, %RPs, and CHF (because it was statistically significant between the groups), and the only variable that predicted low-response to both aspirin and clopidogrel was %RPs (p = 0.02). With multiple linear regression analysis with the same variables, expression of GP IIb/IIIa and P-selectin were also significantly predicted only with %RPs (p = 0.03 for both). Percent RPs also predicted LTA in response to AA (p = 0.003) and ADP (p = 0.02). Interestingly, age also significantly predicted LTA response to AA (p = 0.001). The MPV and aspirin dose, alone or in addition to %RPs, did not predict low response to antiplatelet therapy or platelet activation.

Discussion

We have demonstrated for the first time that platelets from patients with stable CAD and elevated platelet turnover, reported by the proportion of circulating RPs, exhibit significantly increased aggregation and activation responses even after dual antiplatelet therapy. The number of patients with suboptimal or “low response” to aspirin or clopidogrel or both was clustered among patients with a high proportion of circulating RPs. We have previously reported an association between increased platelet reactivity and the proportion of circulating RPs. We have previously reported an association between increased platelet reactivity and the proportion of circulating RPs. We have previously reported an association between increased platelet reactivity and the proportion of circulating RPs.
reported rates of aspirin resistance with other modalities but is higher than reported rates with AA (31,32), although the reasons for this are unclear. One possibility is that we had an older population with known CAD, which in our study significantly predicted responses to AA by regression analysis, consistent with other published studies (33).

Reticulated platelets are recently formed platelets that receive their name due to their similarity to red blood cell reticulocytes and the patterns of distribution of messenger ribonucleic acid that produces a reticulated pattern after staining with thiazole orange. The proportion of circulating RPs is increased in experimental models of enhanced thrombopoiesis and is increased in patients with diseases characterized by increased platelet turnover, such as bleeding and idiopathic thrombocytopenic purpura (11,30,34). Younger platelets are believed to be more active physiologically (16,35) and are also increased in acute coronary syndromes and stroke (12,13). Thus, they are more likely to participate in thrombosis. Their role in modulating the responses to antiplatelet medications such as aspirin and clopidogrel are not known. We have described the impact of young platelets in modifying these responses, to suggest that elevated platelet turnover or RPs could decrease responses to dual antiplatelet therapy. It is possible that young platelets are more reactive (35), and that aspirin and clopidogrel in the doses currently administered do not adequately suppress the activity of young platelets. Aspirin’s half-life in the plasma is short, and once-a-day aspirin might not suffice to adequately inhibit newly formed platelets in a high turnover state. In addition, it is possible that new platelets could regenerate COX-1 and P2Y12 receptors for activation. They might produce enough thromboxane A2 or have enough receptors for cross-linking and activation to be physiologically relevant, as suggested by GP IIb/IIIa expression and platelet aggregometry in our study. We have previously demonstrated that elevated RPs are also associated with increased thromboxane synthesis, with inadequate suppression of COX-1 (14). In addition, non–COX-1–mediated processes, such as through COX-2, have also been suggested to play a role in thromboxane synthesis in young platelets (14,36,37).

We have also demonstrated an association between platelet size and platelet activation after antiplatelet therapy. When assessed by flow cytometry, larger platelets had significantly greater activation patterns compared with smaller platelets after antiplatelet therapy. Some studies have suggested that increased platelet size independently modifies platelet function irrespective of age (38). In our study, most of the RPs (>60%) were in the large platelet group and comprised >15% of all large platelets compared with <2% in the small platelet group. In addition, although our study is not sufficiently powered to test for differences in platelet function with respect to MPV, regression analysis with platelet size using MPV did not predict responses to antiplatelet therapy in addition to %RPs. Thus, platelet size could just be a surrogate for young platelets, and we cannot conclude from this study that large platelets modify responses to antiplatelet therapy independent of %RPs. This study does provide evidence that large platelets are more active and could potentially have an independent role in modifying the effects of antiplatelet therapy, although this will need to be studied further in later studies.

Aspirin dose did not seem to make any difference in platelet responses to aspirin. When patients in each tertile of RPs were stratified into either an 81- or a 325-mg aspirin dose group, the dose strength did not affect the antiplatelet effects of aspirin. Differences between the tertiles persisted despite the higher aspirin dose, suggesting that an aspirin dose >81 mg administered once daily would not overcome the inability of aspirin to adequately inhibit platelets in patients with a higher platelet turnover (RPs). Whether aspirin or clopidogrel administered in multiple daily doses provides more effective platelet inhibition in patients with a high platelet turnover remains to be evaluated.

Study limitations. The main limitation of this study is the inability to assess clinical outcomes due to the sample size. Larger studies might be required to ascertain clinical relevance and importance. Further mechanistic studies are necessary to determine how platelet age and size modify platelet function after antiplatelet therapy. Absolute platelet size of the large and small platelet gate could not be obtained, because the flow cytometer was not equipped to accurately provide these measures. Antiplatelet therapy efficacy with respect to MPV has to be studied in larger patient populations, because our study was not powered to study this.

Conclusions

We demonstrate that an elevated proportion of young platelets and possibly large platelets are associated with increased platelet reactivity in patients with stable CAD who are being treated with dual antiplatelet therapy with both aspirin and clopidogrel. Aspirin dose, when taken once
daily, does not seem to make a difference in platelet responses when stratified by proportion of young platelets.

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REFERENCES


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