Clinical Investigation and Reports

Platelet Glycoprotein IIIa $P_{IIA}$ Polymorphism, Fibrinogen, and Platelet Aggregability
The Framingham Heart Study

DaLi Feng, MD; Klaus Lindpaintner, MD; Martin G. Larson, SD; Christopher J. O’Donnell, MD; Izabella Lipinska, PhD; Patrice A. Sutherland, BS; Murray Mittleman, MD; James E. Muller, MD; Ralph B. D’Agostino, PhD; Daniel Levy, MD; Geoffrey H. Tofler, MD

Background—Recent data suggest that the $P_{IIA}$ allele of the platelet glycoprotein IIIa receptor may be a genetic risk factor for cardiovascular disease. We previously reported that the $P_{IIA}$ allele was associated with increased platelet aggregability, as indicated by lower epinephrine threshold concentrations. Paradoxically, however, it has been reported that $P_{IIA}$-positive platelets have reduced fibrinogen binding. Because fibrinogen mediates platelet aggregability, we hypothesized that plasma fibrinogen levels may interact with $P_{IIA}$ genotype in modulating platelet aggregability.

Methods and Results—Glycoprotein IIIa $P_{IIA}$ genotype, fibrinogen level, and platelet aggregability were ascertained in 1340 subjects enrolled into the Framingham Offspring Study. Platelet aggregability was evaluated by the Born method. Higher fibrinogen levels were associated with increased epinephrine-induced aggregation ($P=0.002$) and a trend for ADP-induced aggregation ($P=0.07$). The fibrinogen effect was genotype specific, however, in that the increase in platelet aggregability with higher fibrinogen was present for the $P_{IIA/A1}$ genotype ($P=0.0005$ and $P=0.03$ for epinephrine- and ADP-induced aggregation, respectively) but not for the $P_{IIA}$-positive genotype ($P>0.90$).

Conclusion—Higher fibrinogen levels were associated with increased platelet aggregability. However, the association between fibrinogen and platelet aggregability was genotype specific. This interaction may be responsible for the conflicting findings regarding $P_{IIA}$ genotype and platelet aggregability. Further study of this gene-environment interaction may provide insight into cardiovascular disease risk. (Circulation. 2001;104:140-144.)

Key Words: platelets $|$ genetics $|$ glycoproteins $|$ fibrinogen

Myocardial infarction results from the formation of a platelet-rich thrombus at the site of a ruptured coronary atherosclerotic plaque. The platelet surface receptor glycoprotein IIb/IIIa (GP IIb/IIIa) plays a key role in the formation of such thrombi by binding with fibrinogen and other ligands, including von Willebrand factor (vWF). The importance of the GP IIb/IIIa receptor has been further supported by recent clinical trials in which GP IIb/IIIa antagonists have been shown to reduce the morbidity and mortality associated with unstable angina, high-risk coronary angioplasty, and acute myocardial infarction.

Weiss and colleagues first reported that patients with acute coronary syndromes were more likely to carry the $P_{IIA}$ variant of the GP IIIa receptor than were controls. The risk associated with $P_{IIA}$ was especially high for those aged $\leq 60$ years at the time of infarction. Recently Walter and colleagues reported that patients with the $P_{IIA}$ variant had an increased risk of coronary stent thrombosis compared with $P_{IIA}$-homozygous individuals. In addition, $P_{IIA}$ has been associated with restenosis after coronary stent placement. However, an association between the $P_{IIA}$ and cardiovascular disease (CVD) has not been a consistent finding.

Recently, we found that the presence of the $P_{IIA}$ variant was associated with increased platelet aggregability as indicated by a lower epinephrine threshold concentration. There was also a trend toward $P_{IIA}$ being associated with a decreased threshold concentration for ADP, which was directionally consistent with the results seen with epinephrine-induced aggregation. Similar results have been reported with thrombin receptor–activating peptide. Paradoxically, however, Goldschmidt-Clermont et al showed that platelets with the $P_{IIA}$ variant bound less exogenous fibrinogen than did the $P_{IIA}$-homozygous platelets when stimulated with ADP. Fibrinogen mediates platelet aggregation by binding to
GP IIb/IIIa, and fibrinogen levels influence platelet aggregability.\textsuperscript{15-17} In addition, vWF is a glycoprotein that mediates platelet adhesion by binding with platelet surface receptor GP IIb/IX. This receptor is particularly important in hemostasis and thrombosis at high shear rate. vWF also binds with the platelet GP IIb/IIIa receptor, mediating platelet aggregation. In the current study, we hypothesized that platelet GP IIIa PlA\textsuperscript{a} genotypes may interact with plasma fibrinogen in modulating platelet aggregability. We also evaluated whether plasma vWF levels were associated with platelet aggregability, and if so, whether PlA\textsuperscript{a} genotypes may interact with vWF levels in modulating platelet aggregability.

### Methods

#### Study Population

The study subjects were members of the Framingham Offspring Study, a long-term, prospective evaluation of risk factors for CVD. The study was approved by the Beth Israel Deaconess Medical Center institutional review committee, and all subjects signed a written informed consent form. The design and methodology of the Framingham Offspring Study have been described in detail elsewhere.\textsuperscript{18} The participants are natural or adopted children of the original Framingham Heart Study subjects. For this study, we collected data from consecutive subjects examined between April 3, 1991, and June 29, 1995, during the fifth Offspring Study examination cycle. Of the 3799 subjects who attended examination cycle 5, blood samples were collected from 3286 subjects for platelet aggregation analysis. For the present analysis, we excluded subjects who were not members of a sibship (n = 1298) because linkage analysis was also performed.\textsuperscript{19} We also excluded subjects who were receiving an anticoagulant, aspirin, or other antiplatelet drug (n = 536), subjects in whom genotyping could not be successfully accomplished (n = 30), and subjects in whom fibrinogen levels were not measured (n = 82). A total of 1340 subjects fulfilled all inclusion criteria.

#### Determination of Platelet Aggregability, Fibrinogen, and vWF Levels

Blood samples were obtained in the morning to avoid circadian variation in platelet aggregability.\textsuperscript{19} Blood was drawn in 3.8% sodium citrate solution (9:1 vol/vol). Platelet-rich plasma was separated by centrifugation for 10 minutes at 160g. Platelet aggregation was measured according to the method of Born.\textsuperscript{20} The aggregation agents tested were epinephrine and ADP in varying concentrations (0.01 to 30 \(\mu\text{mol/L}\)) and a fixed concentration of arachidonic acid (1.6 \(\mu\text{mol/L}\)). The lowest concentrations of ADP and epinephrine required to produce a biphasic response with a lower threshold concentration indicates an increase in platelet aggregability. Therefore, a lower threshold concentration indicates an increase in platelet aggregability.

Blood for fibrinogen was drawn into 3.8% sodium citrate (9:1, vol/vol), and blood for vWF was collected in EDTA. Plasma was separated by centrifugation for 20 minutes at 2000g and stored at \(-80^\circ\text{C}\) for later analysis. Fibrinogen was determined by the Clauss method. vWF antigen was measured by ELISA. The intra-assay coefficients of variation for fibrinogen and vWF in our laboratory were 2.6% and 8.8%, respectively, and the inter-assay coefficients of variation were 4.7% and 10.6%, respectively.

#### Genotyping

Detailed methods have been reported previously.\textsuperscript{11,21} Polymerase chain reaction results were scored without knowledge of platelet aggregation results. Genotyping was successful in 97% of the samples.

### Statistical Analysis

Demographic and clinical characteristics were compared by Student’s \(t\) tests or \(\chi^2\) tests between genotype groups defined by the presence or absence of the PlA\textsuperscript{A2} allele. Observed genotype frequencies were compared with the Hardy-Weinberg equilibrium prediction, also using \(\chi^2\) tests. Data on epinephrine and ADP threshold concentrations were log-transformed. Multiple linear regression models were used to analyze platelet aggregability, adjusting for age, sex, body mass index, diabetes, triglycerides, HDL cholesterol, and estrogen replacement status, together with the presence or absence of the PlA\textsuperscript{A2} allele and fibrinogen quartile (determined separately for each sex).\textsuperscript{22,23} Generalized estimating equation algorithms were used to correct for intra-family correlation.\textsuperscript{24} Data on platelet aggregation were expressed as geometric mean ± 95% CI.

### Results

#### Subject Characteristics

The characteristics of the study subjects are shown in Table 1. Subject characteristics with and without the PlA\textsuperscript{A2} allele were quite similar, with the exception that cigarette smoking was slightly less common among the PlA\textsuperscript{A2}-positive genotype subjects (16% versus 20%, \(P = 0.04\)). Fibrinogen levels were also similar between the 2 groups. The allele frequencies of PlA\textsuperscript{A1} and PlA\textsuperscript{A2} were 0.85 and 0.15, respectively. Genotype frequencies were 71.7% for the PlA\textsuperscript{A1}/PlA\textsuperscript{A2} genotype, and 2.5% for the PlA\textsuperscript{A2}/PlA\textsuperscript{A2} genotype. The genotype frequencies are in accord with the Hardy-Weinberg equilibrium (\(P = 0.65\)).

#### Plasma Fibrinogen Levels and Platelet Aggregability

Increased plasma fibrinogen levels were associated with incrementally increased platelet aggregability for epinephrine-induced aggregation and a trend for ADP-induced aggregation, as indicated by decreases in the threshold concentrations for epinephrine and ADP (\(P = 0.002\) and 0.07, respectively) (Table 2), after adjusting for covariates and the presence of PlA\textsuperscript{A2} allele.

### Table 1. Sample Characteristics

<table>
<thead>
<tr>
<th>Subject Characteristics</th>
<th>PlA\textsuperscript{A1}/PlA\textsuperscript{A1}</th>
<th>PlA\textsuperscript{A1}/PlA\textsuperscript{A2} or PlA\textsuperscript{A2}/PlA\textsuperscript{A2}</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>961</td>
<td>379</td>
<td>...</td>
</tr>
<tr>
<td>Sex, % male</td>
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<td>46</td>
<td>0.98</td>
</tr>
<tr>
<td>Age, y</td>
<td>53±10</td>
<td>54±11</td>
<td>0.45</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>33</td>
<td>29</td>
<td>0.13</td>
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<tr>
<td>Diabetes, %</td>
<td>6</td>
<td>6</td>
<td>0.85</td>
</tr>
<tr>
<td>Smoker, %</td>
<td>20</td>
<td>16</td>
<td>0.04</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>27.5±5.1</td>
<td>27.8±5.3</td>
<td>0.27</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.67±1.22</td>
<td>1.58±0.97</td>
<td>0.16</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.28±0.96</td>
<td>5.31±0.93</td>
<td>0.69</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.27±0.39</td>
<td>1.30±0.39</td>
<td>0.30</td>
</tr>
<tr>
<td>Alcohol, oz/wk</td>
<td>2.9±4.2</td>
<td>2.4±3.7</td>
<td>0.07</td>
</tr>
<tr>
<td>Menopause, %</td>
<td>66</td>
<td>62</td>
<td>0.29</td>
</tr>
<tr>
<td>Estrogen therapy, %</td>
<td>12</td>
<td>11</td>
<td>0.90</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>302±58</td>
<td>305±57</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD or percentages.
Interaction Between Plasma Fibrinogen and $P_{II}^A$ Genotype on Platelet Aggregability

The threshold concentrations for epinephrine-stimulated aggregation decreased with higher fibrinogen levels among the $P_{II}^A$-homozygous subjects, with mean epinephrine threshold concentrations of 1.11 (range, 0.95 to 1.30) μmol/L in the lowest fibrinogen quartile, 1.02 (0.87 to 1.19) μmol/L in the second quartile, 0.85 (0.72 to 1.00) μmol/L in the third quartile, and 0.72 (0.60 to 0.86) μmol/L in the highest fibrinogen quartile (trend $P=0.0005$) (Figure 1). However, the threshold concentrations were not related to fibrinogen levels among the $P_{II}^{A2}$-positive genotype, with respective mean epinephrine threshold concentrations of 0.67 (0.52 to 0.86) μmol/L, 0.81 (0.63 to 1.04) μmol/L, 0.80 (0.62 to 1.05) μmol/L, and 0.63 (0.48 to 0.83) μmol/L (trend $P=0.91$). The interaction term describing the fibrinogen-related alterations in epinephrine threshold concentrations between genotypes was of borderline statistical significance ($P=0.06$, multivariate analyses).

For ADP-induced aggregation, the threshold concentrations also decreased with increased fibrinogen levels in the $P_{II}^A$-homozygous group, with ADP threshold concentrations of 3.20 (range, 3.02 to 3.39) μmol/L, 3.18 (3.01 to 3.37) μmol/L, 2.97 (2.80 to 3.16) μmol/L, and 2.89 (2.70 to 3.09) μmol/L from the lowest quartile to the highest quartile (trend $P=0.03$) (Figure 2). As seen with epinephrine-induced platelet aggregation, there was no relation found between fibrinogen level and ADP threshold concentration in the subjects with $P_{II}^{A2}$-positive genotype. The respective ADP threshold concentrations were 2.83 (2.58 to 3.10) μmol/L, 2.97 (2.71 to 3.26) μmol/L, 3.17 (2.87 to 3.49) μmol/L, and 2.88 (2.61 to 3.19) μmol/L (trend $P=0.97$). The interaction term assessing fibrinogen-related differences in ADP-induced aggregation between genotypes was statistically significant ($P=0.05$, multivariate analyses).

Similar results were obtained when we analyzed the relation between fibrinogen levels and platelet aggregability and the interaction between fibrinogen and $P_{II}^A$ genotype on platelet aggregability with fibrinogen as a continuous variable (data not shown).

Platelet aggregation thresholds were modestly but statistically significantly correlated between siblings for epinephrine threshold concentration ($r=0.245, P=0.0001$) and for ADP threshold concentration ($r=0.235, P=0.0001$). We obtained very similar results for the regression interaction terms relating $P_{II}^A$ genotype with fibrinogen quartile to aggregation thresholds whether the sibship information was included or excluded (data not shown).

There was no significant association between plasma vWF antigen levels and platelet aggregation induced by ADP or epinephrine ($P=0.70$ and 0.96, respectively; multivariate analyses). Furthermore, vWF did not modify the association between the $P_{II}^A$ genotype and platelet aggregability (data not shown).

### Discussion

In a prior Framingham Heart Study investigation, we reported that the $P_{II}^{A2}$ allele of the platelet GP IIIa receptor was associated with increased platelet aggregability. In this investigation, we found that higher fibrinogen levels were associated with increased platelet aggregability. In addition, there was an interaction between the $P_{II}^A$ genotype and fibrinogen levels such that the association of greater platelet aggregability with increased fibrinogen levels was significant for the $P_{II}^{A0/A1}$ genotype but not for the $P_{II}^{A2}$-positive genotype. Despite these differences, subjects with the $P_{II}^{A2}$-positive genotype...
genotype had an overall higher platelet aggregability compared with the $P_{IA}^{A1}$-homozygous subjects.

Studies of twins suggest an important pathogenic role for genetic factors in the pathogenesis of CVD.\textsuperscript{25} Although a small proportion of CVD can be attributed to a single gene defect, the nature of additional contributing genetic factors and their interaction with environmental factors remains largely unknown. Because platelets play a central role in acute CVD, it is possible that inherited platelet receptor variants and their interaction with environmental factors may contribute to the onset of CVD.

**GP IIIa Polymorphism, Fibrinogen, and Platelet Aggregability**

Platelet GP IIb/IIIa is the most abundant platelet receptor.\textsuperscript{26} The receptor is highly polymorphic and has long been recognized as having alloantigens, which cause immune-mediated platelet destruction. We found that one variant of these polymorphisms, the $P_{IA}^{A2}$ allele, was associated with heightened platelet aggregability as indicated by a lower epinephrine threshold concentration.\textsuperscript{11} Although these findings were supported by studies with thrombin receptor-activating peptide (TRAP)\textsuperscript{12,13} and ADP,\textsuperscript{27} the association between the $P_{IA}^{A3}$ and increased platelet aggregability has not been consistent. For example, Lasne et al.\textsuperscript{28} found that platelets of $P_{IA}^{A2}$-positive genotype needed higher concentrations of TRAP to induce platelet dense granule secretion. Goldschmidt-Clermont et al.\textsuperscript{24} quantified exogenous fibrinogen binding to platelets of different genotypes. Paradoxically, these investigators found that platelets with the $P_{IA}^{A2}$ allele bound less exogenous fibrinogen than did platelets that were homozygous for $P_{IA}^{A1}$. However, using the same method, Meiklejohn et al.\textsuperscript{29} found that the percentage of platelets positive for fibrinogen binding was similar among the $P_{IA}^{A2}$-positive platelets and the $P_{IA}^{A1}$-homozygous platelets.

The reason for the difference in platelet reactivity associated with the $P_{IA}^{A1}$ genotype among the various studies is uncertain. The difference is not caused by altered platelet receptor density.\textsuperscript{27,30} One possible explanation is that the mild platelet aggregability change produced by the genetic variation could be masked when a large fixed concentration of agonist is used.\textsuperscript{29} In addition, studies with small sample sizes may not have the power to detect differences.\textsuperscript{31} In comparison, in our large sample, we titrated the platelet agonists to obtain threshold concentration.\textsuperscript{11}

In the present study, increased plasma fibrinogen levels in the physiological range were associated with increases in platelet aggregability as indicated by reductions of epinephrine and ADP threshold concentrations. Prior studies similarly reported that an increase of fibrinogen either in the physiological range in the plasma or added extrinsically was associated with a reduction of the median effective dose of ADP.\textsuperscript{15,16} These findings are consistent with fibrinogen’s role as ligand for the GP IIb/IIIa receptor. In addition, increased fibrinogen concentrations have also been shown to enhance ADP- and TRAP-induced platelet P-selectin expression.\textsuperscript{32} It is therefore possible that platelet reactivity associated with the $P_{IA}^{A}$ polymorphism may be modulated by plasma fibrinogen levels.

**Plasma Fibrinogen Levels Interact With $P_{IA}^{A}$ Polymorphism on Platelet Aggregability**

Our results show an interaction between the $P_{IA}^{A}$ genotype and fibrinogen levels on platelet aggregability such that the enhanced platelet aggregability with increased fibrinogen levels were observed in the $P_{IA}^{A1/A1}$ genotype but not in the $P_{IA}^{A2}$-positive genotype. However, the $P_{IA}^{A2}$-positive genotype still had an overall higher platelet aggregability compared with the $P_{IA}^{A1}$-homozygous even though the $P_{IA}^{A2}$-positive genotype platelets bind a smaller\textsuperscript{14} or a similar\textsuperscript{29} amount of exogenous fibrinogen with the GP IIb/IIIa receptor than do the $P_{IA}^{A1/A1}$ platelets. Our findings partially explain the conflicting findings regarding the $P_{IA}^{A2}$ allele and platelet function.\textsuperscript{11,14,27,29,31} Plasma fibrinogen level should be taken into account when platelet reactivity studies are interpreted because fibrinogen level is an important determinant for platelet reactivity and a modulator of platelet aggregability associated with the $P_{IA}^{A}$ genotype.\textsuperscript{15,16,32}

The mechanism for this interaction is unknown. The $P_{IA}^{A}$ antigen system is not in the 2 putative RGD (Arg-Gly-Asp)-binding regions of the GP IIIa ($P_{IA}^{A1}$ is a leucine and $P_{IA}^{A2}$ a proline at amino acid position 33). However, according to Calvete,\textsuperscript{33} the $P_{IA}^{A}$ system could be brought into immediate proximity with the binding regions by 2 disulfide bonds. Because of the unique structure of proline and its propensity for inducing conformational change, the amino acid substitution could influence the affinity of GP IIb/IIIa receptor for fibrinogen. Thus, the platelet with the $P_{IA}^{A2}$ variant might have an increased binding with endogenous plasma fibrinogen at basal conditions. If that is the case, fewer GP IIb/IIIa receptors will be available for exogenous fibrinogen after being stimulated as reported by Goldschmidt-Clermont et al.\textsuperscript{14} Further study of endogenous and exogenous fibrinogen binding is needed to test the hypotheses. Alternatively, platelets with the $P_{IA}^{A2}$ allele may have intrinsically increased aggregability as indicated by a lower epinephrine threshold concentration and therefore may be less dependent on plasma fibrinogen levels to achieve a similar degree of platelet aggregation as measured by the Born method.\textsuperscript{11,13}

**Strengths and Limitations of the Study**

In a large number of subjects, we obtained genotype status, fibrinogen levels, and platelet aggregability data to evaluate the relation between the $P_{IA}^{A}$ genotype and platelet aggregability and the interaction between the genotype and fibrinogen on platelet aggregability. A detailed risk factor profile was collected and adjusted for potential confounders. However, this cross-sectional study had several limitations. First, our analysis was based on the subset of Framingham subjects in whom both genotype and phenotype data were available. However, the genotype distribution and subject characteristics were similar among subjects excluded from analysis or included in the present analysis (data not shown). Second, we used an in vitro method to evaluate the relation between the $P_{IA}^{A}$ polymorphism, fibrinogen levels, and platelet aggregability. The in vivo significance of our in vitro findings and their clinical relevance need to be evaluated further. Finally, we used epinephrine and ADP threshold concentrations to determine platelet aggregability. Many different methods and
parameters have been used to evaluate platelet aggregability, which may influence the results. For example, in one study, increased fibrinogen concentrations led to increased platelet fibrinogen bonding but reduced aggregation velocity. Meade et al. found that increased fibrinogen levels were associated with a reduced median effective dose of ADP, a finding similar to our current result, indicating increased platelet aggregability. However, they also reported that increased fibrinogen was associated with a lower maximal response when higher ADP concentration was used, suggesting decreased aggregability.

Implications of the Study

We found that increased plasma fibrinogen levels were associated with increased platelet aggregability, although this association was present for the PlA1A1 but not the PlA2 genotype. Prospective studies are warranted to examine the combined effect of fibrinogen levels and the PlA polymorphism on incidence of CVD. If individuals with the PlA2 variant (who have increased platelet aggregability irrespective of fibrinogen levels) or PlA2 homozygotes with high fibrinogen levels have a higher incidence of CVD, they may benefit from more aggressive measures for prevention and treatment, including therapy with antiplatelet agents.

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