Novel Platelet Membrane Glycoprotein VI Dimorphism Is a Risk Factor for Myocardial Infarction

S.A. Croft, BSc; N.J. Samani, MD, FRCP; M.D. Teare, PhD; K.K. Hampton, MD, MRCP, MRCPath; R.P. Steeds, MA, MRCP; K.S. Channer, MD, FRCP; M.E. Daly, PhD

**Background**—Glycoprotein (GP) VI plays a crucial role in platelet activation and aggregation. We investigated whether polymorphic variation at the GP VI locus confers an increased risk of myocardial infarction (MI).

**Methods and Results**—Coding and 5′ and 3′ non-coding regions of the GP VI gene were analyzed by polymerase chain reaction and conformation sensitive gel electrophoresis in 21 healthy subjects. Ten dimorphisms, 5 of which predicted amino acid substitutions (T13254C, A19871G, A21908G, A22630T, C22644A), were identified. Two core haplotypes involving 7 dimorphisms (C10781A and G10873A and all those predicting amino acid substitutions) were apparent. The contribution of the T13254C dimorphism, which predicted the substitution of serine 219 by proline, to risk of MI was assessed in 525 patients with acute MI and 474 controls, all aged $\geq 75$ years. The allelic odds ratio (OR) for MI associated with the 13254C allele was 1.16 (95% CI, 0.91 to 1.46; $P=0.23$). Compared with corresponding control subgroups, the 13254CC genotype was more common among cases who were female (OR, 4.52; 95% CI, 1.23 to 16.64; $P=0.029$), nonsmokers (OR, 2.50; 95% CI, 0.98 to 6.38; $P=0.048$), aged $\geq 60$ years (OR, 6.48; 95% CI, 1.47 to 28.45; $P=0.009$) or carried the β-fibrinogen -148T allele associated with increased fibrinogen levels (OR, 10.49; 95% CI, 1.32 to 83.42; $P=0.02$). In logistic regression analysis that took other cardiovascular risk factors into account, the interactions of GP VI genotype with age ($P=0.005$) and β-fibrinogen genotype ($P=0.035$) remained significant.

**Conclusions**—The GP VI 13254CC genotype increases the risk of MI, particularly in older individuals, and the interaction of the GP VI 13254C allele with other candidate risk alleles may accentuate this risk. (Circulation. 2001;104:1459-1463.)

Key Words: myocardial infarction ▪ genetics ▪ platelets

Several platelet receptors for collagen are recognized; glycoproteins (GP) Ia/IIa and VI are of primary importance. GP Ia/IIa is required for platelet adhesion to collagen, whereas subsequent changes in tyrosine phosphorylation and activation of phospholipase $\mathrm{C}_\gamma 2$ are mediated through GP VI.1,2 We recently showed that the 807T allele of the GP Ia gene that is associated with increased GP Ia/IIa expression and platelet adhesiveness to collagen did not confer an increased risk of myocardial infarction (MI) and proposed that polymorphic variation at the GP VI locus might contribute to an increased risk of bleeding or thrombosis under certain circumstances.3 In the present study, analysis of the GP VI gene sequence in a panel of healthy subjects resulted in identification of 10 GP VI gene dimorphisms. The effect of one of these dimorphisms on risk of MI was then assessed.

**Subjects**
Peripheral blood samples were obtained for GP VI gene analysis, after receiving informed consent, from 21 healthy volunteers working in the Royal Hallamshire Hospital, Sheffield, UK. GP VI T13254C genotypes were determined for 525 MI patients who survived to admission to the Cardiac Care Units of Leicester Royal Infirmary and the Royal Hallamshire Hospital (Sheffield) and who satisfied the World Health Organization criteria for MI. The 474 control subjects were healthy visitors to patients with noncardiovascular illnesses recruited at both the Leicester Royal Infirmary and the Royal Hallamshire Hospital. Control subjects with a history of angina or MI were excluded from analysis. All subjects were aged $<75$ years and of white origin. Further details of recruitment were described previously.3 The study received local ethics committee approval in both centers.

**See p 1451**

**Methods**
Peripheral blood samples were obtained for GP VI gene analysis, after receiving informed consent, from 21 healthy volunteers working in the Royal Hallamshire Hospital, Sheffield, UK. GP VI T13254C genotypes were determined for 525 MI patients who survived to admission to the Cardiac Care Units of Leicester Royal Infirmary and the Royal Hallamshire Hospital (Sheffield) and who satisfied the World Health Organization criteria for MI. The 474 control subjects were healthy visitors to patients with noncardiovascular illnesses recruited at both the Leicester Royal Infirmary and the Royal Hallamshire Hospital. Control subjects with a history of angina or MI were excluded from analysis. All subjects were aged $<75$ years and of white origin. Further details of recruitment were described previously. The study received local ethics committee approval in both centers.
GP VI Gene Analysis

Using GP VI cDNA, the GP VI genomic sequence was identified in the human genome database and assembled from 2 chromosome 19 contigs (accession numbers AC019238 and AC011476).

Polymerase chain reaction (PCR) was used to amplify DNA fragments corresponding to all exons, flanking intronic sequences, and 5' and 3' non-coding regions of the GP VI gene from 21 healthy volunteers. Reactions containing 25 ng of genomic DNA and 50 ng of the appropriate primers were prepared and subjected to cycling conditions using an annealing temperature of 58°C, as previously described. PCR products were then subjected to conformation sensitive gel electrophoresis to detect heteroduplexes. In all cases in which heteroduplexes were detected, PCR product was purified and directly sequenced. Alterations in the GP VI gene sequence were confirmed where possible by restriction digestion of the corresponding PCR product.

GP VI T13254C and β-Fibrinogen -148C/T Genotyping

GP VI T13254C genotypes were determined by PCR amplification of a 279-bp fragment encompassing exon 5 of the GP VI gene using primers 5'-ACATCCACCAACAGTCCAGTG (forward) and 5'-ATCGAGAAGTCTAGGCAGAG (reverse), as indicated, followed by HpaII digestion of the product. Digestion resulted in fragments of 120 bp, 112 bp and 47 bp in the presence of the T-allele or 112 bp, 95 bp, 47 bp, and 25 bp in the presence of the C allele. The -148C/T dimorphism in the β-fibrinogen gene was genotyped by amplification and HindIII digestion of a 495-bp fragment spanning nucleotides 2388 to 107 of the β-fibrinogen gene.

Statistical Analysis

The χ2 test was used to compare GP VI T13254C genotype distributions, allele frequencies, and qualitative risk factors between cases and controls. One-way ANOVA was used to compare quantitative risk factors, and 95% confidence intervals (CIs) for allele frequencies were calculated using the normal distribution approximation. Odds ratios (OR) with 95% CIs were calculated to estimate relative risk of MI associated with carriership of the GP VI 13254C allele. Multivariate logistic regression analysis was performed using SPSS Version 10, and the statistical significance of any variable or model was evaluated using the likelihood ratio test.

Results

GP VI gene analysis in 21 healthy subjects identified 9 coding sequence dimorphisms and 1 dimorphism located 154 bp away from the translation start codon. The GP VI gene analysis results are shown in Figure 1.

**Figure 1.** Schematic representation of GP VI gene, mRNA, and protein. A, Exons (black) and introns (white) of GP VI gene showing positions of dimorphisms and any predicted amino acid substitutions. Dimorphisms contributing to the core haplotype are boxed, and the common alleles are indicated in bold. Nucleotides and amino acids are numbered relative to the translation start codon. B, GP VI mRNA indicating size of each exon. C, GP VI protein showing location of amino acid dimorphisms relative to putative signal peptide (SP), mucin-like (MR), and transmembrane (TM) domains. D, Sequences, at dimorphic sites, of GP VI cDNAs deposited with GenBank and published by Jandrot-Perrus et al and genotypes for the dimorphisms contributing to the core haplotype (indicated in bold) observed in the present study.

**TABLE 1.** Demographic Details of Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>Sheffield</th>
<th></th>
<th>Leicester</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
<td>Controls</td>
</tr>
<tr>
<td>Age, y</td>
<td>61.9±9.2</td>
<td>61.1±9.1</td>
<td>61.5±9.3*</td>
<td>54.4±11.8</td>
</tr>
<tr>
<td>Males, %</td>
<td>64.7</td>
<td>62.7</td>
<td>72.9*</td>
<td>59.3</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>29.7*</td>
<td>16.4</td>
<td>34.1*</td>
<td>15.9</td>
</tr>
<tr>
<td>Mean total cholesterol, mmol/L</td>
<td>6.0±1.2</td>
<td>5.9±1.0</td>
<td>5.7±1.3</td>
<td>5.6±1.0</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>8.5*</td>
<td>1.4</td>
<td>9.4*</td>
<td>3.3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.9±4.0</td>
<td>25.8±3.7</td>
<td>25.9±4.0</td>
<td>25.5±3.7</td>
</tr>
<tr>
<td>Current smokers, %</td>
<td>41.3*</td>
<td>15.8</td>
<td>41.5*</td>
<td>18.7</td>
</tr>
</tbody>
</table>

Data are mean±SD or percent. BMI indicates body mass index.

*P<0.01.
4bp upstream of the translation start site (Figure 1). Five dimorphisms predicted amino acid substitutions. Two core haplotypes involving 7 dimorphisms (C10781A, G10873A, T13254C, A19871G, A21908G, A22630T, and C22644A), including all those predicting amino acid substitutions, were apparent (Figure 1). The existence of 3 of these dimorphisms could be predicted by comparing the 5 GP VI cDNA sequences published or deposited in GenBank to date, which reveals identical sequences in 4 cases, whereas the fifth (gi13651089) differs at nucleotide positions 22644, 22630, and 21908 (Figure 1). Of the 21 subjects studied, 13 were homozygous and 6 were heterozygous for the 10781C/10873G/13254T/19871A/21908A/22630A/22644C haplotype. Two subjects had different genotypes, as indicated in Figure 1.

To investigate whether polymorphic variation at the GP VI locus may be associated with risk of MI, 525 MI cases and 474 controls from Sheffield and Leicester were genotyped for the T13254C dimorphism (Figure 2). This dimorphism was selected because it contributed to the core GP VI gene haplotype and because the resulting substitution of serine 219 by proline might be predicted to influence GP VI structure-function relationships. Table 1 shows the demographic details of the cohorts. Although the Sheffield cases and controls showed no difference in age and sex, the Leicester cases were older and had a greater proportion of men than the controls (Table 1). Hypertension, diabetes mellitus, and smoking were more prevalent among cases than controls in both centers, but there was no difference in mean body mass index or total cholesterol level between case and control groups at either center.

There was no significant difference in T13254C genotype distribution among either cases (P=0.40) or controls (P=0.12) in Sheffield and Leicester, which allowed results to be combined for analysis purposes (Table 2). The 13254C allele frequency among combined cases was 0.18 (95% CI, 0.16 to 0.21) compared with 0.16 (95% CI, 0.14 to 0.18) among controls, yielding an allelic OR for MI of 1.16 (95% CI, 0.91 to 1.46; P=0.23). The 13254CC genotype seemed to be more prevalent among cases (4.0%) than controls (2.1%), although this difference was not significant (CC versus T carrier: P=0.12). Subgrouping according to sex revealed that although GP VI T13254C genotype was not associated with risk among men, homozygosity for the 13254C allele increased MI risk ~4-fold among women (P=0.02). The 13254CC genotype was also associated with an increased risk of MI among nonsmokers (OR, 2.50; 95% CI, 0.98 to 6.38; P=0.048) and among subjects aged ≥60 years (OR, 6.48; 95% CI, 1.47 to 28.45; P=0.009). There was no association between T13254C genotype and MI risk in subgroups selected according to the presence or absence of hypertension or diabetes mellitus or among cases having a cholesterol level or a body mass index either less or greater than the median for the control groups (data not shown).

We examined whether possession of both the GP VI 13254C allele and the GP Ia 807T allele studied earlier could increase risk of MI. The OR for MI in those with the 13254CC genotype compared with those with the 13254TT genotype was 2.34 (95% CI, 0.81 to 6.74; P=0.11) among subjects carrying at least one GP Ia 807T allele (Table 3). Given that plasma fibrinogen increases with age and is positively associated with vascular risk, we also examined whether possession of both the GP VI 13254C allele and the -148T allele of the β-fibrinogen gene promoter, which is associated with elevated fibrinogen levels, could contribute to risk of MI. Although the -148T allele was not an independent risk factor for MI in our population (results not shown), the GP VI 13254CC genotype increased MI risk 10-fold among subjects who also carried the -148T allele (OR, 10.49; 95% CI, 1.32 to 83.42; P=0.02; Table 3).

Logistic regression analysis was performed to assess the significance of the studied genotypes in the presence of other recognized cardiovascular risk factors (Table 4). Significant associations were found between GP VI genotype and age (P=0.005) and between GP VI and β-fibrinogen genotypes.

### Table 2. GP VI T13254C Genotypes in Cases and Controls

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th></th>
<th>Controls</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
<td>TT</td>
<td>CC</td>
</tr>
<tr>
<td>Sheffield</td>
<td>11 (3.8)</td>
<td>89 (30.8)</td>
<td>189 (65.4)</td>
<td>3 (1.0)</td>
</tr>
<tr>
<td>Leicester</td>
<td>10 (4.2)</td>
<td>60 (25.4)</td>
<td>166 (70.3)</td>
<td>7 (3.8)</td>
</tr>
<tr>
<td>Total</td>
<td>21 (4.0)</td>
<td>149 (28.4)</td>
<td>355 (67.6)</td>
<td>10 (2.1)</td>
</tr>
</tbody>
</table>

Values are n (%).

### Table 3. Univariate ORs for MI Associated With the GP VI 13254 CC and CT Genotypes Compared With the TT Genotype for Various Subgroups

<table>
<thead>
<tr>
<th></th>
<th>CT</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>1.04 (0.79–1.38)</td>
<td>1.96 (0.91–4.22)</td>
</tr>
<tr>
<td>Men</td>
<td>0.98 (0.69–1.39)</td>
<td>1.16 (0.43–3.09)</td>
</tr>
<tr>
<td>Women</td>
<td>1.21 (0.76–1.92)</td>
<td>4.52 (1.23–16.64)*</td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>1.01 (0.66–1.56)</td>
<td>0.59 (0.17–2.01)</td>
</tr>
<tr>
<td>≥60 years</td>
<td>1.05 (0.72–1.53)</td>
<td>6.48 (1.47–28.45)*</td>
</tr>
<tr>
<td>Current smokers</td>
<td>0.88 (0.51–1.54)</td>
<td>0.84 (0.21–3.36)</td>
</tr>
<tr>
<td>Current nonsmokers</td>
<td>1.03 (0.74–1.45)</td>
<td>2.50 (0.98–6.38)*</td>
</tr>
<tr>
<td>GP Ia 807T carriers</td>
<td>1.03 (0.73–1.45)</td>
<td>2.34 (0.81–6.74)</td>
</tr>
<tr>
<td>GP Ia 807CC carriers</td>
<td>1.09 (0.68–1.74)</td>
<td>1.57 (0.51–4.83)</td>
</tr>
<tr>
<td>β-Fibrinogen –148T carriers</td>
<td>0.97 (0.60–1.56)</td>
<td>10.49 (3.22–38.43)*</td>
</tr>
<tr>
<td>β-Fibrinogen –148CC carriers</td>
<td>1.10 (0.78–1.56)</td>
<td>1.08 (0.44–2.65)</td>
</tr>
</tbody>
</table>

Values are ORs (95% CIs).

*Significant P value (P<0.05) derived by comparison with the 13254TT genotype: women, P=0.02; subjects aged ≥60 years, P=0.009; current nonsmokers, P=0.048; β-fibrinogen –148T carriers, P=0.02.
The functional significance of polymorphic variation at the GP VI locus remains to be established. We used reverse transcription and PCR amplification of platelet RNA to confirm expression of GP VI mRNA derived from both alleles in subjects heterozygous for the T13254C dimorphism (results not shown). Further studies will determine whether this dimorphism is directly associated with or linked to differences in the expression or stability of GP VI mRNA. Of particular interest is the -154C/T dimorphism located upstream of the translational start site that could be associated with differential GP VI gene expression. It is also possible that the Ser219Pro substitution predicted by the T13254C dimorphism is directly or indirectly through linked amino acid substitutions, associated with differences in GP VI function. Interestingly, the linked dimorphism A21908G predicts a substitution of threonine 249 by alanine in the mucin-like domain of GP VI that is rich in serine and threonine residues and may be involved in O-linked glycosylation, a process that in other receptors is thought to facilitate interactions with bulky ligands and protect against proteolysis.4

In conclusion, we have shown that polymorphic variation at the GP VI locus is associated with susceptibility to MI and that this may be accentuated by gene-gene interactions involving GP VI and other candidate risk alleles. Our findings suggest that therapeutic strategies targeting GP VI should be considered as approaches for controlling platelet reactivity and in the prevention and treatment of cardiovascular disease.

Discussion

In this study, we found that homozygosity for the C-allele of the T13254C dimorphism was more common among MI cases than controls and conferred 2- and 4-fold increases in risk of MI among nonsmokers and women, respectively, although this was not confirmed by logistic regression analysis that allowed for other risk factors. More strikingly, the association between GP VI 13254CC genotype and vascular risk was strongest among subjects aged 60 years ($P=0.035$). Tests for interactions between GP VI genotype and sex, smoking, or GP Ia genotype were not statistically significant. The ORs estimated from the logistic regression were consistent in magnitude and direction with those shown in the univariate analysis for all variables examined (not shown).

Heterozygosity for the GP VI 13254C allele was not associated with an increased MI risk in any subgroup studied, indicating that the presence of one 13254T allele compensated for the prothrombotic effects of the 13254C allele. The 10-fold increase in risk of MI associated with possession of both the 13254CC genotype and the -148T allele of the $\beta$-fibrinogen gene indicates that although the latter is not an independent risk factor for MI, its interaction with the 13254CC genotype may be sufficient to confer an increase in overall MI risk.

The functional significance of polymorphic variation at the GP VI locus remains to be established. We used reverse transcription and PCR amplification of platelet RNA to confirm expression of GP VI mRNA derived from both alleles in subjects heterozygous for the T13254C dimorphism (results not shown). Further studies will determine whether this dimorphism is directly associated with or linked to differences in the expression or stability of GP VI mRNA. Of particular interest is the -154C/T dimorphism located upstream of the translational start site that could be associated with differential GP VI gene expression. It is also possible that the Ser219Pro substitution predicted by the T13254C dimorphism is directly or indirectly through linked amino acid substitutions, associated with differences in GP VI function. Interestingly, the linked dimorphism A21908G predicts a substitution of threonine 249 by alanine in the mucin-like domain of GP VI that is rich in serine and threonine residues and may be involved in O-linked glycosylation, a process that in other receptors is thought to facilitate interactions with bulky ligands and protect against proteolysis.4

In conclusion, we have shown that polymorphic variation at the GP VI locus is associated with susceptibility to MI and that this may be accentuated by gene-gene interactions involving GP VI and other candidate risk alleles. Our findings suggest that therapeutic strategies targeting GP VI should be considered as approaches for controlling platelet reactivity and in the prevention and treatment of cardiovascular disease.

References


Novel Platelet Membrane Glycoprotein VI Dimorphism Is a Risk Factor for Myocardial Infarction
S. A. Croft, N. J. Samani, M. D. Teare, K. K. Hampton, R. P. Steeds, K. S. Channer and M. E. Daly

*Circulation*. 2001;104:1459-1463
doi: 10.1161/hc3801.096397

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/104/13/1459

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/