P2Y<sub>12</sub> H2 Haplotype Is Associated With Peripheral Arterial Disease

A Case-Control Study

Pierre Fontana, MD; Pascale Gaussem; Martine Aiach; Jean-Noël Fiessinger; Joseph Emmerich; Jean-Luc Reny, MD

Background—We recently described a gain-of-function haplotype, called H2, of the adenosine diphosphate (ADP) receptor P2Y<sub>12</sub> gene associated with increased ADP-induced platelet aggregation ex vivo in healthy volunteers. Because platelets play a key role in atherosclerosis and arterial thrombosis, we tested the possible link between the H2 haplotype and the risk of peripheral arterial disease (PAD) in a case-control study.

Methods and Results—We studied 184 consecutive male patients under 70 years of age with PAD and 330 age-matched control subjects free of symptomatic PAD and with no cardiovascular history. Mean age was 57.1±7.2 years (cases) and 56.7±7.6 years (control subjects). The H2 haplotype was more frequent in patients with PAD than in control subjects (30% and 21%, respectively; OR, 1.6; CI, 1.1 to 2.5; P=0.02 in univariate analysis). This association with PAD remained significant in multivariate regression analysis (OR, 2.3; CI, 1.4 to 3.9; P=0.002) after adjustment for diabetes, smoking, hypertension, hypercholesterolemia, and other selected platelet receptor gene polymorphisms.

Conclusions—These data point to a role of the H2 haplotype in atherosclerosis and raise the possibility of relative thiopyridine resistance in carriers of the P2Y<sub>12</sub> H2 haplotype. (Circulation. 2003;108:2971-2973.)

Key Words: atherosclerosis ■ arteries ■ platelets ■ thrombosis ■ peripheral vascular disease

Platelet aggregation is a key event in arterial thrombosis. It is also involved in the initiation and development of atherosclerotic lesions, through platelet adhesion to dysfunctional endothelium and release of growth factors and cytokines. Adenosine diphosphate (ADP) belongs to the key mediators of platelet stimulation and mediates its effect through two 7-transmembrane receptors, P2Y<sub>1</sub> and P2Y<sub>12</sub>. P2Y<sub>12</sub> plays a particularly important role in platelet aggregation, since its coupling to a Gi protein is responsible both for stabilizing platelet aggregates and for amplifying aggregation induced by ADP and other agonists. The importance of the ADP receptor P2Y<sub>12</sub> is emphasized by the fact that patients with cardiovascular disease derive a greater benefit when it is blocked by thienopyridines than when platelet function is inhibited by aspirin. The P2Y<sub>12</sub> receptor gene was recently cloned, and we identified polymorphisms defining two haplotypes designated H1 and H2; we found that H2 acted as a gain-of-function haplotype on ex vivo ADP-induced aggregation of platelets from healthy volunteers.

Atherosclerotic disease mainly targets cerebral, coronary, and peripheral arteries. Peripheral arterial disease (PAD) is associated with a high risk of coronary and cerebrovascular events. Epidemiological studies show that 75% of patients with PAD die of vascular causes. In the Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) study, P2Y<sub>12</sub> receptor blockade by clopidogrel was particularly beneficial in atherosclerotic patients with PAD compared with patients with a history of myocardial infarction (MI) or ischemic stroke. Given this potential specificity of the P2Y<sub>12</sub> receptor in patients with PAD and the gain of function conferred by the H2 haplotype in platelet aggregation, we sought a possible link between the H2 haplotype and PAD in a case-control study. We also genotyped subjects for α<sub>IIb</sub>β<sub>3</sub> PL<sub>AluA2</sub> and α<sub>c</sub>β<sub>3</sub> 807C/T, two other well-characterized platelet receptor gene polymorphisms.

Methods

Study Population

Cases were consecutive white male patients with PAD under the age of 70 years, recruited in a vascular medicine department in Paris. They had symptomatic PAD of the lower limbs, with either an ankle-brachial systolic pressure index (ABI) of <0.9 or a history of surgical or endovascular revascularization. They were ineligible if they had nonatherosclerotic causes of PAD (cardioembolic disease, thromboangiitis obliterans, vasculitis, or congenital or metabolic vascular diseases). Control subjects had no history of arterial disease (stroke, MI, angina, or PAD) and were randomly selected, with age matching, among 703 white men of a previously described control population.
group used to study genetic risk factors for vascular thrombosis. They were recruited in a Parisian health center specializing in screening for cardiovascular diseases. Cases and control subjects were screened for the following vascular risk factors by means of a medical questionnaire, blood pressure measurements, and laboratory analyses: (1) smoking (current, previous, or never); (2) diabetes mellitus (fasting blood glucose $>7$ mmol/L, or blood glucose-lowering treatment); (3) hypertension (resting arm systolic blood pressure $>140$ mm Hg or diastolic blood pressure $>90$ mm Hg or antihypertensive treatment); (4) hypercholesterolemia (plasma LDL cholesterol $>4.1$ mmol/L or lipid-lowering treatment). Over a 2-year period, 184 cases were enrolled and were matched with 330 control subjects (146 cases had 2 control subjects each, whereas 38 cases had only 1 control subject each). All participants gave written informed consent, and the Paris-Cochin Ethics Committee approved the study protocol.

Genotyping of P2Y$_{12}$ H2 haplotype was performed with the use of a polymerase chain reaction (PCR) method targeting the i-T744C single nucleotide polymorphism with 5’-TCACTTATCTCGAGGGAAATGAAGAGATACGTA-3’ (sense primer) and 5’-GGTCAAGAAATGGCCTGTATATATGGTCATGAGTTGGCGT-TCACTTATC-ACC-3’ (antisense primer). The thermal cycling conditions comprised an initial denaturation step at 95°C for 5 minutes and 38 cycles at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. A final extension step was performed at 72°C for 7 minutes. The PCR product was then digested with Rsal and analyzed by gel electrophoresis. Genotyping of $\alpha_\text{IIb} \beta_\text{3}$ PL$^{A382}$ and $\alpha_\text{IIb} \beta_\text{3}$ 807C/T gene polymorphisms were performed as previously described.\cite{10,11}

Statistical Analysis
The characteristics of the subjects are presented as means and standard deviations for continuous variables and as counts and percentages for categorical variables. Groups were defined as cases and control subjects. To detect an odds ratio of $\geq 2$, with an $\alpha$-risk of 0.05 and a $\beta$-risk of 0.20, we calculated that 150 cases and 300 control subjects were required, based on the reported allelic frequency of the P2Y$_{12}$ H2 haplotype.\cite{6} Comparisons between groups were made with Student’s unpaired $t$ test for continuous variables and a $\chi^2$ or Fisher’s exact test for categorical variables. The association between the presence of the H2 allele and PAD was tested, after adjustment for other variables, by using multivariate logistic regression analysis.

Results
Table 1 summarizes the characteristics of the cases and control subjects. The two groups differed significantly in terms of smoking status, hypertension, and diabetes. The percentage of hypercholesterolemic patients was similar in the two groups, but significantly more cases were receiving lipid-lowering drugs; total and LDL cholesterol levels were therefore lower in the cases. The prevalence of traditional vascular risk factors in the cases was similar to that in other studies.\cite{4} Most of the cases had intermittent claudication, and 31% had coronary artery or ischemic cerebrovascular disease.

Thirty per cent of cases had at least one H2 allele, compared with 21% of control subjects ($P=0.02$). The H2 allele was associated with PAD in univariate analysis, with an OR of 1.6 (Table 2). As expected, in the absence of extreme population stratification for other risk factors, the multivariate analysis did not affect the univariate results. Indeed, after adjustment for common cardiovascular risk factors (hypertension, hypercholesterolemia, diabetes, and smoking status), the OR rose to 2.2 (CI, 1.3 to 3.6; $P=0.003$). Further adjustment with PL$^{A382}$ and 807C/T polymorphisms did not significantly change the magnitude of this association, with the OR slightly increasing to 2.3 (CI, 1.4 to 3.9; $P=0.002$) (Table 2). There was no significant association between the P2Y$_{12}$ polymorphism and a history of ischemic events. The frequencies of the PL$^{A382}$ and 807T alleles in cases and control subjects were, respectively, 0.15/0.17 ($P=0.7$) and 0.38/0.37 ($P=0.9$), values again within the range of previously reported

<table>
<thead>
<tr>
<th>TABLE 1. Characteristics of the Study Population</th>
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<td>Cases (n=184)</td>
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<tr>
<td>Age, y</td>
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<td>Current past smokers, %</td>
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<td>Hypertension, %</td>
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<td>Fasting blood glucose, mmol/L</td>
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<td>Asymptomatic, %</td>
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<tr>
<td>Intermittent claudication, %</td>
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<td>Critical ischemia, %</td>
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<td>Prior revascularization, %</td>
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<td>Ankle-brachial systolic pressure index</td>
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<td>Coronary heart disease, %</td>
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<td>Cerebrovascular disease, %</td>
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Values are given as mean±SD or percentages. BMI indicates body mass index.

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These latter data contribute to validation of our control group.

**Discussion**

With regard to the possible limitations of this study, ABI was not determined in the control subjects, which possibly led to some misclassifications. However, misclassification of asymptomatic subjects with PAD in the control group would have tended to bias the results toward the null hypothesis. As in all genetic association studies, the use of small populations increases the risk of false-positive findings. Thus, our hypothesis-generating data require further confirmation from independent studies and in other situations such as MI and ischemic stroke.

Analysis of platelet aggregation induced by various platelet agonists indicates that the ADP receptor P2Y12 acts as a hub in the platelet activation process. Together with the benefit conferred by its blockade in clinical studies, a gain-of-function haplotype of the P2Y12 gene potentially influences diseases in which platelets play a major role. Indeed, this is the first study showing a significant association between this functional P2Y12 gene polymorphism and atherosclerosis. Moreover, our findings raise the possibility of relative thienopyridine resistance in carriers of the P2Y12 H2 haplotype.

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**References**

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