Determinants of Plasma Levels of Plasminogen Activator Inhibitor-1
A Study of Normotensive Twins

Maurizio Cesari, Maria Teresa Sartori, Giovanni M. Patrassi, Silvia Vettore, Gian Paolo Rossi

Abstract—We investigated whether plasma levels of the plasminogen activator inhibitor type 1 antigen (PAI-1:Ag) are genetically determined in monozygotic (MZ) and dizygotic (DZ) twins. Twenty-five pairs of healthy twins underwent measurements of PAI-1:Ag and other variables, including body mass index, mean blood pressure, plasma renin activity, insulin, and glucose. To ascertain the zygosity of twins, highly discriminating micro- and minisatellite systems with variable numbers of tandem repeats were analyzed by PCR amplification followed by polyacrylamide gel electrophoresis. Subjects were also genotyped for the 4G/5G polymorphism by PCR. Estimates of genetic variance and heritability were obtained for PAI-1:Ag, and for body mass index, mean blood pressure, plasma renin activity, glucose, and insulin by jointly examining data in a path analysis with TWINAN90. Results showed that 12 pairs of twins were MZ and 13 were DZ. All tests of genetic variance [within pair (WP): F = 6.24, P = 0.002; among component (AC): F = 2.62, P = 0.04; average absolute difference t test = 3.00, P = 0.004] showed significant genetic variance of PAI-1:Ag, but not of the other variables. Three tests of heritability (WP = 0.837, P = 0.002; AC = 1.791, P < 0.05; intraclass correlation: 1.180, P = 0.001) consistently showed significant PAI-1:Ag heritability. Additive genetic influences (A), dominance genetic effect (D), and random environmental influences (E) accounted for 0.714, 0.154, and 0.132 of PAI-1:Ag variance, respectively. No effect of different 4G/5G genotypes was found. Thus, these results show significant genetic variance and heritability of PAI-1:Ag and suggest that A is more important than both D and E in determining PAI-1:Ag variance. (Arterioscler Thromb Vasc Biol. 1999;19:316-320.)

Key Words: plasminogen activator inhibitor type 1 • fibrinolysis • gene polymorphism • twins • humans

Plasminogen activator inhibitor type 1 (PAI-1) is the key regulator of the activity of the fibrinolytic system, an important protective mechanism against thrombosis. Reduced fibrinolytic activity, mainly caused by increased plasma levels of PAI-1, is a common finding in many cross-sectional studies of patients with coronary artery disease (CAD) and recurrent myocardial infarction (MI), deep vein thrombosis, and stroke. Although extensive investigations have led to identification of the environmental factors regulating PAI-1:Ag and PAI-1 activity (PAI-1:act) levels in plasma, only limited information exists on their genetic determinants. To our knowledge the following 8 polymorphisms of the PAI-1 gene have been identified: two (CA)n repeat polymorphisms, a HindIII restriction fragment length polymorphism, an insertion (5G)/deletion (4G) polymorphism on the promoter, ie, −675 bp upstream from the start of transcription of the PAI-1 gene, and 4 polymorphisms in the PAI-1 gene by use of the single-strand conformational polymorphism (SSCP) method. A major effect of the 4G/5G polymorphism on PAI-1:act was reported, but conflicting results are also available. In fact, a relationship between both PAI-1 antigen and activity levels and the 4G/5G polymorphism (the 4G homozygous having the highest mean plasma PAI-1 levels) has been shown in patients with CAD, non-insulin-dependent diabetes mellitus (NIDDM), and healthy control subjects. However, in contrast with these findings no significant association was found between both PAI-1 antigen and activity levels and the 4G/5G polymorphism or the 4G homozygous having the highest mean plasma PAI-1 levels. This issue is of major clinical relevance, and the results of genetic studies of twins are increasingly popular in molecular genetics, because the comparison of monozygotic (MZ) and dizygotic (DZ) twins offers a unique and powerful method of partitioning genetic and environmental sources of covariance of quantitative traits which may be relevant for late-onset diseases, such as atherosclerosis, hypertension, and related cardiovascular disease. They can also provide estimates of the additive (A) genetic effects, which combine independently over different alleles at the same genetic locus and
over different loci, dominant (D) effect, ie, allelic interactions at the same locus, the environmental effects common to cotwins (C, ie, shared family environment), and the environmental (E) variance unique to individuals on the variance of quantitative traits such as the components of the clotting cascade. Therefore, this study was undertaken to investigate the genetic and environmental components of the plasma levels of PAI-1 in identical and fraternal normotensive twins.

Subjects and Methods

Twin

The twins investigated in this study were enrolled from an Italian association of twins based in Padua, Italy. To ascertain whether twins were MZ or DZ, some highly discriminating micro- and minisatellite systems with a variable number of tandem repeats were analyzed. Three amplified fragment-length polymorphisms (Apob, D1S80, and YNZ22) and 4 short tandem repeats (HUMACTPB2, HUMTH01, HUMFES/FP5, and HUMMVA31) were assessed by separate PCR amplifications of genomic DNA followed by polyacrylamide gel electrophoresis and silver staining. No subject was smoking or taking any drugs or medications known to affect the fibrinolytic tests for at least 15 days before the study. None participated in any competitive sports training or reported daily intake of alcohol. All subjects were healthy and showed normal fasting levels of serum glucose and insulin. Blood pressure (BP) was measured with a mercury sphygmomanometer using Korotkoff phase V for diastolic and according to the WHO guidelines. All gave an informed written consent, and the Institutional Human Subjects Review Committees of the University of Padua Medical School approved the study protocol.

Blood Sampling and Biochemical Measurements

Blood was collected after an overnight fast and 15-minute supine rest. Blood was sampled from 100 mL Na2EDTA 6% was immediately put on ice. After centrifugation at 3000g (4°C for 10 minutes), separated aliquots of plasma and buffy coat were stored at −40°C and tested within 1 month. Plasma renin activity was measured by a commercially available kit (Ares Seronno, Milan, Italy, supine normal values with a month. Plasma renin activity was measured by a commercially manufacturer’s instructions. Genomic DNA was amplified with allele-specific primer pairs, using the Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn) with the following conditions: 94°C, 5 minutes; 94°C, 1 minute, 72°C, 2 minutes for 5 cycles; 94°C, 1 minute, 65°C, 45 seconds, 72°C, 1 minute 15 seconds for 25 minutes for 1 cycle; 94°C, 1 minute, 72°C, 2 minutes for 5 cycles; 96°C, 5 minutes for 1 cycle. The PCR products were run in TBE 0.5× buffer in a 4% agarose gel stained with ethidium bromide.

Statistical Analysis

Results are expressed as mean±SD, or SEM, as appropriate.20 Comparison of subjects of the different genotypes was carried out with Kruskall–Wallis test for plasma renin activity, or with a 1-way ANOVA followed by Bonferroni’s multiple comparison test for the variables that showed a normal distribution. Because subjects in each twin pair could not be regarded as independent unrelated individuals, either twin 1 or twin 2 of each pair was considered for statistical comparison. Analysis was carried out with the SPSS for Windows™ statistical package (version 7.5, SPSS Inc, Chicago, Ill). A P value <0.05 was considered statistically significant. Analysis of twin data was carried out jointly with TWINAN90 (a generous gift of Prof. C.J. Williams), a program specifically developed for conducting analyses of twin data.21 To this end the consistency of the variable of interest with the normal distribution assumption and with the hypothesis of equal variance between zygosity was verified beforehand. Estimates of genetic variance, including the within-pair (WP) and among components (AC), were attained thereafter, and a test for genetic variance based on the average absolute difference between twins,22 which is standardized to yield an approximate t test for the null hypothesis of no genetic variance, was performed. Three estimates of heritability were also calculated; the first two were derived from the WP and AC estimates of genetic variance, respectively, according to the following equations:

\[
WP = 2(W_{MZ} - W_{DZ})/(S_{MZ} + S_{DZ})^2
\]

\[
AC = (AC)^2/(S_{MZ} + S_{DZ})^2
\]

The third estimate of heritability (h^2) is based on intrapair correlation coefficients calculated from the MZ and DZ twins as follows:

\[
h^2 = 2(r_{MZ} - r_{DZ})
\]

This approach allows the determination of the proportion of variance of PAI-1:Ag accounted for by an additive genetic influence (A) which combines independently over different alleles at the same locus (C), or a nonadditive genetic influence (D) (ie, allelic interactions at the same locus), environmental influences shared by cotwins within a family (C) and unique to individuals (E).21 This ANOVA-based method was chosen because it was more accurate than path-maximum-likelihood methods to estimate and test the significance of A because it does not require the assumption that C=0.22 Finally, the results of the likelihood-based analyses were determined for models with A, D, and E (ADE); A, C, and E (ACE); A and E (AE); C and E (CE), and E. This type of analysis was carried out also for body mass index (BMI), mean blood pressure (MBP), glycemia, and plasma fasting insulin.

Results

After ascertainment of zygosity, 12 twins were determined to be MZ and 13 DZ. The observed 4G/5G PAI-1 genotype distribution was in agreement with the Hardy–Weinberg proportion (4/4=0.14, 4/5=0.54, 5/5=0.32) in the whole population, as well as in the MZ and DZ subgroups (all P=NS by χ2 test). Their demographic and biochemical characteristics, including PAI-1:Ag levels, are shown in Table 1. No significant difference between MZ and DZ twins and among different 4G/5G PAI-1 genotypes in all variables of interest was found (Table 1).

Joint analysis of the MZ and DZ twins data was carried out for the variable PAI-1:Ag with TWINAN90.21 A Kolmogorov–Smirnov goodness-of-fit test applied separately to the grouped pairs for each of the two zygosities confirmed that PAI-1:Ag levels followed a normal distribution (P>0.15 for both zygosities), and therefore no transformation of the data was needed. The null hypothesis that the mean of the MZ and DZ twins absolute difference did not differ from zero was thereafter accepted (t=1.62, with 18.7 degree of freedom, NS) and the equal variance hypothesis was not rejected (F=0.61, NS). Estimates of...
genetic variance and intraindividual correlation coefficients for both MZ and DZ twins were obtained, and a test for genetic variance was performed (Table 2). Both the preferred tests of genetic variance WP and average absolute difference tests and also the AC estimate resulted in being statistically significant. The three estimates of heritability were also calculated (Table 2), and the results were being statistically significant. The three estimates of heritability were not statistically significant for serum glucose, insulin, and mean blood pressure. Statistically significant correlation coefficients for mean blood pressure and serum glucose, both in MZ and DZ twins, and in either MZ or DZ twins (for body mass index and serum insulin, respectively) were found. However, all tests of genetic variance and heritability were not statistically significant for serum glucose, insulin, and mean blood pressure. Only BMI showed a statistically significant AC genetic variance and heritability ($P=0.011$) and a borderline significant intraindividual correlation coefficient ($P=0.042$).

### Discussion

In this study, to gain insight into the relative effects of genes and environment on PAI-1:Ag, we compared the plasma levels of PAI-1:Ag of fraternal (DZ) and identical (MZ) twins selected from a Caucasian population, who were young, healthy, and normotensive and had normal plasma levels of PAI-1:Ag. Their zygosity status was carefully established both at the phenotypic and at the molecular level by assessing some highly discriminating variable number of tandem repeats micro- and minisatellite systems. In addition utmost care was also taken to ensure an accurate measurement of PAI-1:Ag both during sample collection and at assay. Data were analyzed with a strategy selected because it takes into account that PAI-1:Ag levels are heritable.

The genetic variance was estimated to be $51.7 \pm 72.6$ ng/mL, and the heritability was $0.714$. The non-genetic variance was estimated to be $11.15 \pm 67.4$ ng/mL, and the proportion of non-genetic variance was $0.154$. The overall error variance was estimated to be $9.55 \pm 4.1$ ng/mL, and the proportion of error variance was $0.132$.

#### Table 2: Output of Path Analysis Carried Out With TWINAN90

<table>
<thead>
<tr>
<th>PAI-1:Ag</th>
<th>Estimate</th>
<th>Variance</th>
<th>F Ratio</th>
<th>P Value</th>
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</thead>
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<tr>
<td><strong>Genetic variance</strong></td>
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<td></td>
<td></td>
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<tr>
<td>WP</td>
<td>63.50</td>
<td>783.6</td>
<td>6.24</td>
<td>0.002</td>
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<td>AC</td>
<td>135.9</td>
<td>24,583.5</td>
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<td><strong>Intrapair correlations</strong></td>
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<tr>
<td>MZ</td>
<td>0.935</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
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<tr>
<td>DZ</td>
<td>0.345</td>
<td>0.106</td>
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<tr>
<td><strong>Average absolute difference</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>t test</td>
<td>3.00</td>
<td>17.3</td>
<td>0.004</td>
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<td><strong>Heritability</strong></td>
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<tr>
<td>WP</td>
<td>0.837</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC</td>
<td>1.791</td>
<td>0.040</td>
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<td><strong>Intraclass correlations</strong></td>
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<td>1.180</td>
<td>0.001</td>
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<td><strong>ADE model</strong></td>
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<td>Additive genetic variance (A)</td>
<td>51.7</td>
<td>72.6</td>
<td>0.714</td>
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<tr>
<td>Dominance genetic variance (D)</td>
<td>11.15</td>
<td>67.4</td>
<td>0.154</td>
<td></td>
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<tr>
<td>Error variance (E)</td>
<td>9.55</td>
<td>4.1</td>
<td>0.132</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Scatter plot of WP plasma levels of PAI-1:Ag with regression lines and 95% confidence intervals. MZ (closed circles) and DZ (open circles) twins are shown. The correlation was significant ($P<0.001$) in MZ but not in DZ twins.
consideration the contribution of shared environment and therefore allows investigation of the proportion of variance of PAI-1:Ag accounted for by an additive genetic influence (A), nonadditive genetic influences (D) (ie, allelic interactions at the same locus), environmental influences shared by cotwins within a family (common, C) and unique to individuals (E). At variance with a recent study of Swedish twins investigating the genetic influences of PAI-1:act with a multivariate genetic technique (LISREL), we used TWINAN90, an ANOVA-based method specifically developed for conducting analyses of twin data by Williams et al, because this approach was found to be more accurate than path-maximum-likelihood methods for estimating and testing the significance of A.22

By using this strategy, we found strong evidence of genetic variance and heritability of PAI-1:Ag in these subjects (Table 2). When phenotypic covariance was partitioned into additive genetic effects (A), dominant genetic components (D), environmental effects common to cotwins (C), and error variance (E) components, the estimates indicated that more than 85% was accounted for by A and D (Table 2), thereby suggesting that most of PAI-1:Ag variance is genetically determined. This contention is also supported by the finding of high and statistically significant correlation coefficients only in MZ and not in DZ twins. These results concerning PAI-1:Ag are in sharp contrast with those concerning other demographic, biochemical, and hemodynamic variables, which are of potential interest for cardiovascular risk, such as fasting serum glucose, insulin, and mean blood pressure.

In a recent study of white nuclear families from a healthy French population, variables related to the insulin resistance syndrome were found to explain a major part of PAI-1 variance (49% in fathers and 29% in mothers), whereas five PAI-1 gene polymorphisms accounted for a negligible proportion (3% in women and none in men) of PAI-1 variability.24 These findings differed from those of a Swedish study of twins in which the proportion of PAI-1:act levels accounted for by heritability and individual-specific environmental factors was 42% and 36%, respectively.23 Although these differences could be caused by several methodological factors, such as measurement of PAI-1:Ag versus PAI-1:act, study design, statistical technique used, in our view the most likely explanation relates to the different selection criteria of the study populations. It is in fact conceivable that our selection of young healthy normotensive twins with normal PAI-1 levels might have led to higher estimates of genetic variance, compared with those studies in which older subjects with higher BMI values and a much wider dispersion of PAI-1 levels were investigated.23,24

The 4G/5G PAI-1 polymorphism has been proposed as a determinant of the plasma levels of PAI-1:Ag. It has also been put forward as a risk factor for acute myocardial infarction in patients with angina pectoris and NIDDM,11,12,25 although the prognostic role of the 4G/5G polymorphism in the development of MI is still under debate.9,26,27 When classified by 4G/5G genotype, our twins were demographically similar and had superimposable blood pressure values (Table 1). The 4G or 5G allele frequency was consistent with the Hardy–Weinberg proportion, both in the whole twins population sample and when the twins were classified according to their zygosity. Although this finding makes a selection bias unlikely and suggests that the present results can be representative of the general population, it must be acknowledged that the observed 4G/5G genotype distribution in our series corresponds with a 4G allele frequency of 0.41, which is somewhat lower than the frequency of 0.54 to 0.56 reported in most studies in Caucasian populations (see for review9,26). Nevertheless, the present results do not support the contention that 4G/5G polymorphism plays a major role in determining PAI-1:Ag, because no significant difference among different 4G/5G genotypes was found.

Were the 4G/5G polymorphism exerting a major effect on PAI-1:Ag, the WP difference of 4G/5G concordant DZ twins would be expected to be close to that of MZ twins and lower than that of 4G/5G discordant DZ twins. In contrast, we found that the WP difference of 4G/5G concordant DZ twins differs significantly from that of MZ and was similar to that of DZ discordant twins (Figure 2). This finding suggests a role of additional, possibly still unidentified polymorphisms on the PAI-1 locus and/or of other quantitative trait loci contributing significantly to PAI-1:Ag, an issue which obviously deserves further specific research.

Figure 2. WP difference of plasma levels of PAI-1:Ag in MZ twins and in DZ twins concordant and discordant for the 4G/5G genotype. A significant difference between MZ twins and 4G/5G concordant DZ twins was found, whereas 4G/5G discordant twins had values similar to those of discordant DZ twins.

### References

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