Age-dependent Prevalence of Vascular Disease-associated Polymorphisms among 2689 Volunteer Blood Donors

MARTIN J. HESSNER,1* DAVID M. DINAUER,2 ROBERT KWIAKTOWSKI,3 BRUCE NERI,3 and THOMAS J. RAIFE2

Background: The development of vascular disease involves the interaction of genetic and environmental factors. Because vascular disease is a major contributor to mortality in Western societies, we hypothesized that deleterious polymorphisms associated with hemostasis decrease in frequency among a healthy population as a function of age.

Methods: The frequencies of factor V G1691A Leiden (FVL), factor II (FII) G20210A, methylenetetrahydrofolate reductase (MTHFR) C677T, glycoprotein Ia (GPIa) C807T, glycoprotein IIIa (PIA1/PIA2) T1565C, and angiotensin-converting enzyme (ACE) intron 16 insertion/deletion (I/D) alleles were determined among 2689 healthy Caucasian whole-blood donors. For analysis, participants were divided into three age groups: 17–39 years (n = 979; 505 males and 474 females), 40–59 years (n = 900; 526 males and 374 females), and 60–85 years (n = 810; 530 males and 280 females).

Results: The PIA2 allele frequency decreased from 17.5% to 15.7% to 14.1% in the 17–39 years, 40–59 years, and 60–85 years age groups, respectively (n = 5094 alleles; P = 0.025). Among ACE DD males, the PIA2 allele frequency decreased from 20.8% to 16.1% and 9.1% in the same groups, respectively (n = 810 alleles; P = 0.001). No statistically significant decrease in genotype or allele frequency was observed among carriers of FVL, FII 20210A, MTHFR 677T, GPIa 807T, or ACE D.

Conclusions: These data suggest that PIA2 carriers, especially those who are ACE DD, are statistically less prevalent among older healthy blood donors compared with their younger counterparts. These observations suggest an important, deleterious, time-dependent impact of the PIA2 allele, as well as the ACE DD/PIA2 allelic combination, on overall health and longevity.

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Vascular disease is a major cause of mortality in Western societies. The development of vascular disease involves the interaction of multiple genetic factors and environmental influences. Numerous polymorphisms have been identified within genes encoding peptides directly and indirectly involved in hemostasis. Many of these genetic markers have been associated with vascular disease in studies evaluating defined patient groups; however, the potential impact of these risk factors, individually and in combination with one another, on overall health and longevity remains unclear. We hypothesized that deleterious alleles, or allelic combinations, will decrease in frequency as a function of population age. Comparison of genotype frequencies among age-stratified healthy populations may be a useful strategy to identify polymorphisms associated with common human diseases, such as vascular disease and cancer. In multifactorial diseases, such as vascular disease, the analysis of large numbers of age-stratified healthy individuals for multiple loci may provide insight into which combinations of high-risk alleles are clinically relevant. To test this hypothesis, the frequencies of the six vascular disease-associated mutations were measured among a cohort of 2689 healthy volunteer Caucasian blood donors.

FACTOR V G1691A
Protein C is a serine protease, which when activated acts as an anticoagulant by inactivating the procoagulant
factors Va (FV) and VIIIa. The FV Leiden (FVL) mutation encodes for substitution of glutamine for arginine at residue 506, abolishing one of three activated protein C cleavage sites in FV (1–3). This mutation has been shown to occur in 20–60% of patients with venous thrombosis and in ~5% of the general population in Europe and the United States (3, 4). Heterozygous carriers of the FV 1691A allele have an ~8-fold increased risk of venous thrombosis; homozygous expression confers an estimated 91-fold increased risk of venous thrombosis (5, 6).

FACTOR II (FII; PROTHROMBIN) G20210A
The conversion of FII to the serine protease thrombin is a key event in thrombosis and hemostasis. A G→A transition at nucleotide 20210 within the 3'-untranslated region of the FII gene is associated with increased plasma prothrombin (7). Heterozygous carriers of the FII 20210A allele, which comprise 1–2% of the general Caucasian population, have an approximately three- to fivefold increased risk of venous thrombosis (5, 8, 9).

METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) C677T
MTHFR catalyzes the NADPH-linked reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as a cofactor in the methylation of homocysteine to methionine. The C677T mutation (Val233 to Ala233) generates a thermolabile enzyme with ~50% of the activity of the wild-type gene product (10, 11). Among different Caucasian populations, the frequency of homozygous individuals ranges from 5% to 16% (5, 11, 12). Hyperhomocysteinemia has been confirmed as a risk factor for venous thrombembolic events and occlusive arterial disease. Homozygosity for the MTHFR 677T allele has been associated with increased plasma homocysteine, and initial reports implicated this genotype as a risk factor for arterial disease. Homozygosity for the MTHFR 677TT genotype is associated with up to 10-fold increased GPIa/IIa receptor on platelet membranes and increased platelet adhesion to type I or type III collagen (17). Because receptor density influences platelet adhesion to collagen, inheritance of the low- or high-density alleles has been postulated to predispose individuals for either bleeding disorders or thromboembolic complications. Patient studies have associated the GPIa 807T allele with increased risk of myocardial infarction, coronary artery disease, and stroke (18–20). Among Caucasians, individuals homozygous for the GPIa 807TT genotype are found at a frequency of ~12%, and the GPIa 807T allele is observed at a frequency of 35–40% (17, 21).

ANGIOTENSIN-CONVERTING ENZYME (ACE) INTRON 16 INSERTION/DELETION
ACE converts vasoactive angiotensin I to the vasoconstrictive angiotensin II and inactivates the vasodilator bradykinin (22). The concentration of plasma ACE within an individual is stable, but there is high variability among individuals. Approximately 50% of this variability is associated with a 287-bp insertion/deletion (I/D) polymorphism within intron 16 of the ACE gene (23). The mean plasma concentration of ACE in D/D homozygotes is approximately twice that of I/I homozygotes, whereas I/D individuals have intermediate circulating ACE concentrations (23). The distributions of the ACE D and I alleles are found at near-equal frequency among healthy Caucasians, giving rise to genotype frequencies of 25%, 50%, and 25% for the II, DI, and DD genotypes, respectively (23, 24). The ACE DD genotype has been controversially identified as a risk factor for myocardial infarction and coronary artery disease (25).

PLATELET GPIIIa T1565C (PlA1/PlA2)
The GPIIb/IIIa complex is the platelet fibrinogen receptor and is the most abundant receptor in the platelet membrane at ~80 000 copies/cell. The PlA2 allelic form, which arises through a single T-to-C nucleotide substitution at nucleotide 1565 of the GPIIa gene and the substitution of proline for leucine at position 33, may produce platelets with a lower activation threshold than PlA2-negative platelets (26–28). The PlA2 allele is observed at a frequency of ~15% in Caucasian populations, with genotype distributions of 71%, 27%, and 1–2% for PlA1/PlA1, PlA1/PlA2, and PlA2/PlA2, respectively. Several studies have examined PlA2 as a risk factor for a wide variety of vascular diseases, particularly coronary artery disease, and have made positive associations (29, 30).

To study whether there is there a difference between the genetic profiles of healthy young individuals and healthy older individuals, 2689 age-stratified healthy Caucasian volunteer blood donors were genotyped for six polymorphisms implicated in the development of vascular disease.

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1 Nonstandard abbreviations: FV, factor V; FVL, factor V Leiden; FII, factor II; MTHFR, methylenetetrahydrofolate reductase; GP, glycoprotein; ACE, angiotensin-converting enzyme; and I/D, insertion/deletion.
Materials and Methods

Peripheral blood was collected in disodium EDTA from 2689 Caucasian healthy whole-blood donors (1561 males and 1128 females) living in the greater Milwaukee, WI area. Approximately 400 samples per each 10-year stratum between the ages of 20 and 80 years were collected. All samples were numbered, unlinked, and tested anonymously. Genomic DNA was isolated from 300 μL ofuffy coat with the QIAamp 96 Blood Kit (Qiagen). DNA was quantified by measuring the absorbance at 260 nm on a SPECTRAmax Plus spectrophotometer (Molecular Devices Corp.), and the concentration was adjusted to 25 mg/L with distilled water.

The FVL G1691A, FII G20210A, and MTHFR C677T genotypes were determined by multiplexed allele-specific PCR as described previously (4). Alternatively, FV G1691A, FII G20210A, and MTHFR C677T were genotyped using the Invader® Assay (Third Wave Technologies, Inc., Madison, WI) (31). GPIa C807T, ACE I/D, and GPIIIa (Pl A1/PlA2) T1565C were genotyped using PCR-based methods described previously (21, 24, 32).

Participants were stratified into three age groups: 17–39 years (n = 979; 505 males and 474 females), 40–59 years (n = 900; 526 males and 374 females), and 60–85 years (n = 810; 530 males and 280 females). The differences in genotype and allele distribution were analyzed statistically using the Pearson $\chi^2$ test, or when appropriate, the Fisher exact test (3, 3, 2 degrees of freedom when comparing genotype frequencies; 2, 3, 2 degrees of freedom when comparing allele frequencies). $P < 0.05$ was considered statistically significant.

Results

The FVL, FII G20210A, MTHFR C677T, GPIa C807T, ACE D/I, and GPIIIa T1565C genotype frequencies and allele frequencies were initially analyzed independently for changes in frequency among the 17–39 years, 40–59 years, and 60–85 years groups. The MTHFR 677T, GPIa 807T, and ACE D alleles are relatively common; therefore, in this study homozygotes for these alleles were observed at a frequency adequate for statistical measurement. The FV G1691AA and FII 20210AA genotypes were not observed; therefore, genotype analysis for these loci evaluated only changes in heterozygous carrier frequency. No significant differences were observed in genotype or allele frequency for FV G1691A, FII G20210A, MTHFR C677T, GPIa C807T, or ACE DD, nor were any significant changes observed when these data were analyzed on the basis of gender. These data are summarized in Table 1.

A decrease in GPIIIa 1565T (Pl A2)-positive genotypes from 32.3% (294 of 911) to 29.4% (252 of 856) to 26.9% (210 of 780) was observed among the 17–39 years, 40–59 years, and 60–85 years groups, respectively; this change was not statistically significant ($2 \times 2 \chi^2$ analysis, 2 degrees of freedom, $P = 0.076$). This change in GPIIIa 1565C carrier frequency in the total group was brought about primarily through a significant decrease in male PlA2 carriers from 32.1% (153 of 477) to 29.4% (148 of 356) to 26.9% (126 of 509) among the 17–39 years, 40–59 years, and 60–85 years groups, respectively ($2 \times 2 \chi^2$ analysis, 2 degrees of freedom, $P = 0.032$). A decrease in the number of Pl A2 homozygotes was also observed, and this change paralleled that of the group of combined carriers. Among the

### Table 1. Genotype and allele frequencies.

<table>
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<tr>
<th></th>
<th>Genotype frequency, %</th>
<th>Total n</th>
<th>$P^a$</th>
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<tbody>
<tr>
<td></td>
<td>17–39 years</td>
<td>40–59 years</td>
<td>60–85 years</td>
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<tr>
<td>FV 1691GA</td>
<td>5.2</td>
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<td>FII 20210GA</td>
<td>3.6</td>
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<td>MTHFR 677TT</td>
<td>10.5</td>
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<td>GPIa 807TT</td>
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<td>15.0</td>
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<td>GPIIIa 1565TC/CC (females)</td>
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<td>29.5</td>
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<table>
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<tr>
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<th>Allele frequency, %</th>
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<th>$P^c$</th>
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<tbody>
<tr>
<td></td>
<td>17–39 years</td>
<td>40–59 years</td>
<td>60–85 years</td>
</tr>
<tr>
<td>FV 1691A</td>
<td>2.6</td>
<td>3.6</td>
<td>2.4</td>
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<tr>
<td>FII 20210A</td>
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<td>1.1</td>
<td>1.3</td>
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<td>MTHFR 677T</td>
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<td>34.0</td>
<td>34.0</td>
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<tr>
<td>GPIa 807T</td>
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<td>39.6</td>
<td>38.9</td>
</tr>
<tr>
<td>ACE D</td>
<td>51.9</td>
<td>54.0</td>
<td>54.6</td>
</tr>
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<td>GPIIIa 1565C</td>
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<td>15.7</td>
<td>14.1</td>
</tr>
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<td>GPIIIa 1565C (males)</td>
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<tr>
<td>GPIIIa 1565C (females)</td>
<td>17.2</td>
<td>15.6</td>
<td>16.2</td>
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</table>

$a$ $3 \times 3 \chi^2$ test, 2 degrees of freedom.

$b$ $2 \times 2 \chi^2$ analysis between 17–39 years group and 60–85 years group: $P < 0.05$.

$c$ $2 \times 3 \chi^2$ test, 2 degrees of freedom.
total group, the number of PlA2 homozygotes decreased from 3.4% (25 of 911) to 2.0% (17 of 856) to 1.3% (10 of 780); among males, PlA2 homozygotes decreased from 3.6% (17 of 477) to 2.2% (11 of 504) and 1.2% (6 of 509). These data are also summarized in Table 1.

To evaluate the possible interaction of multiple risk-associated alleles, every possible combination of two loci was examined, both in terms of genotype frequency and allele frequency. A single statistically significant combination was observed. Among ACE DD males, the decrease in PlA2 carrier frequency was greater than that observed when PlA2 frequency was evaluated as a single variable; in this group, PlA2 carriers decreased from 39.2% (49 of 125) to 29.2% (40 of 137) to 17.5% (25 of 143; 3 × 3 analysis, 2 degrees of freedom, \( P = 0.002 \)). Among ACE DD males, the PlA2 allele frequency decreased from 20.8% (54 of 260) to 16.1% (37 of 230) to 9.1% (17 of 189; 3 × 3 analysis, 2 degrees of freedom, \( P = 0.001 \)). These data are summarized in Table 2.

**Discussion**

In this study, 2689 age-stratified healthy Caucasian blood donors were genotyped for six polymorphisms that have been implicated in the development of vascular disease. The purpose of this study was twofold: (a) to determine whether any of these polymorphisms, either alone or in combination with one another, sufficiently impact health on a population basis such that it can be detected as a decrease in genotype or allele frequency; and (b) to examine a strategy that uses a source of DNA that is relatively easy to obtain as a tool to evaluate the thousands of described human genetic polymorphisms to ascertain which may represent relevant disease markers.

This analysis is based on several assumptions about blood donor populations. Volunteer blood donors are selected on the basis of health and behavioral risk factors for infectious diseases. Donors must be well on the day of donation, not currently under medical care for serious illnesses, and free of high-risk behaviors for specific infectious diseases. We therefore concluded that selection influences among volunteer blood donors are primarily phenotypic influences manifested as disease and mortality and that these phenotypic influences reflect a multitude of underlying genotypic influences that can be discovered by retrospective analysis of successful donors.

Numerous studies have established FV 1691A and FII 20210A as the two alleles that convey the most risk for development of vascular disease among those evaluated in this study, but no significant changes in these frequencies were observed. All FV 1691A and FII 20210A carriers were heterozygous; no homozygous carriers were observed (expected FVL 1691AA frequency, 0.0625%; expected FII 20210AA frequency, 0.014%). Our observation of no age-related frequency decrease in the FV 1691A allele is consistent with the report of Rees et al. (33), in which a cohort of 87 residents of the United Kingdom >90 years of age were found to have a FVL allele frequency similar to that for the general population in the United Kingdom (2.7% vs 2.9%, respectively). It is possible that a measurable frequency change could be detected with a larger sample set. Two FV 1691GA/FII 20210GA compound heterozygotes were observed, and the fact that these two individuals were both in the 17–39 years group (18-year-old female and 37-year-old male) is consistent with the notion that these two risk factors act synergistically in the development of thrombosis (34). Again, a larger sample set would be required to confirm that carriers of this combination of alleles decrease in prevalence in older blood donor populations.

A statistically significant age-associated decrease in the MTHFR 677T allele or 677TT genotype frequency was not observed. Similar to these results, Faure-Delanef et al. (35) reported a 13.3% 677TT frequency among 458 French centenarians vs 18.5% among 374 French controls with a median age of 53.1 years (\( P = 0.12 \)). Likewise, Bladbjerg et al. (36) observed a MTHFR 677TT frequency of 12% among 186 Danish centenarians vs 10% among 201 healthy Danish blood donors with a mean age of 42 years (\( P = 0.74 \)). These results contrast with the findings of Matsushita et al. (37), where the 677TT genotype fre-

**Table 2. ACE DD/PlA2 combination analysis.**

<table>
<thead>
<tr>
<th>Genotype frequency, %</th>
<th>17–39 years</th>
<th>40–59 years</th>
<th>60–85 years</th>
<th>Total n</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE DD/GPIIIa 1565TC/CC</td>
<td>38.0</td>
<td>28.5</td>
<td>25.3</td>
<td>693</td>
<td>0.018</td>
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<td>ACE DD/GPIIIa 1565TC/CC (males)</td>
<td>39.2</td>
<td>29.2</td>
<td>17.5</td>
<td>405</td>
<td>0.002</td>
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<tr>
<td>ACE DD/GPIIIa 1565TC/CC (females)</td>
<td>36.6</td>
<td>27.6</td>
<td>39.7</td>
<td>288</td>
<td>0.262</td>
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</table>

<table>
<thead>
<tr>
<th>Allele frequency, %</th>
<th>17–39 years</th>
<th>40–59 years</th>
<th>60–85 years</th>
<th>Total n</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE DD/GPIIIa 1565C</td>
<td>19.8</td>
<td>15.7</td>
<td>13.4</td>
<td>1386</td>
<td>0.027</td>
</tr>
<tr>
<td>ACE DD/GPIIIa 1565C (males)</td>
<td>20.8</td>
<td>16.1</td>
<td>9.1</td>
<td>810</td>
<td>0.001</td>
</tr>
<tr>
<td>ACE DD/GPIIIa 1565C (females)</td>
<td>18.8</td>
<td>15.3</td>
<td>21.2</td>
<td>576</td>
<td>0.357</td>
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\( a 3 \times 3 \chi^2 \) test, 2 degrees of freedom.

\( b 2 \times 3 \chi^2 \) test, 2 degrees of freedom.
quency decreased from 19% to 7% in healthy Japanese individuals <55 years vs those ≥80 years (P = 0.006).

The ACE D allele and ACE DD genotype frequencies showed no statistically significant age-associated difference in the total group or in males or females. Bladbjerg et al. (36) observed comparable ACE DD frequencies of 27% and 26% for 187 Danish centenarians and 201 healthy Danish blood donors with a mean age of 42 years, respectively (P = 0.97). Agerholm-Larson et al. (38) also reported no change in ACE D gene frequency as a function of age in Danish individuals between the ages of 20 to >80 years. However, Schachter et al. (39) observed a significant increase in the ACE DD genotype among 338 French centenarians compared with adult controls 20–70 years of age (P <0.01).

The evaluation of platelet integrin polymorphisms revealed no frequency changes in the GPIa 807TT genotype or 807T allele, but GPIIIa 1565C/PIA2 carrier frequency decreased as a function of age. Gender subgroup analysis revealed statistically significant decreases in PIA2 carrier and allele frequencies among males and revealed that the overall PIA2 frequency decrease was attributable primarily to changes within the male subgroup (Table 1). The allele frequencies for the three male age groups (17.8–13.0%) bracket the reported 15% PIA2 allele frequency in Caucasians (40). Because the PIA2 allele frequency does vary among different racial groups, a possible difference in racial composition was considered between the young and older donor populations (40). Because PIA2 is less common in non-Caucasian groups and there is likely to be greater racial admixture in the younger donor group, a lower PIA2 frequency would be expected in the younger donor group if this variable was a factor in this study. If the decrease in PIA2 frequency observed in this study is indeed attributable to an effect that this allelic form of GPIIIa has on health and longevity in men, why is there not an effect seen in woman? Recently, Boudoulas et al. (41) reported that aggregation of PIA2 platelets is significantly more inhibited by physiological concentrations of estrogen than aggregation of PIA1 platelets, raising the possibility that different risk factors may be important in men vs woman for the development of vascular disease. In contrast to the results of this study, Bladbjerg et al. (36) observed comparable PIA2 carrier frequencies, 29% vs 29%, for 187 Danish centenarians and 201 healthy Danish blood donors, respectively. In this study, among ACE DD males, the PIA2 carrier frequency decreased as a function of age from 39.2% in the 17–39 years group to 17.5% in the 60–85 years group (P = 0.002). This observation of a relationship between ACE DD and PIA2 is consistent with the finding that the PIA2 and ACE D allelic forms were synergistic for increased risk of recurrent events in patients post-myocardial infarction (42). Lastly, it must be noted that the ACE and GPIIIa genes both map to chromosome 17q21-23; however, no evidence of linkage was observed.

Our findings suggest that PIA2 carriers, especially those who are ACE DD, are less prevalent among older healthy blood donors than among their younger counterparts. This observation raises questions regarding the impact ACE DD/PIA2 allelic combination on overall health and longevity and warrants further study.

The use of age-stratified healthy individuals, such as volunteer blood donors, to study the potential impact of genetic polymorphisms may be a useful approach to the discovery of genetic mutations and polymorphisms that impact health and longevity. Genetic studies that reveal an age-dependent decrease (or increase) of single alleles or allelic combinations can serve as important guides for further biologic studies of the identified factors. Because frequency changes may be subtle, such population studies clearly require the use of very large numbers of participants. To date, most studies have been relatively small. The ongoing development of more efficient automated genotyping platforms and data management systems will facilitate further use of this strategy and potentially demonstrate its utility.

References


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