Frequency of Thrombophilia-Related Genetic Variations in Patients with Idiopathic Pulmonary Embolism in an Urban Emergency Department

Lori Kruse,1 Alice M. Mitchell,1 Carlos A. Camargo, Jr.,2 Jackeline Hernandez,1 and Jeffrey A. Kline1

Background: The frequency of the thrombophilic genetic variants factor V Leiden (FVL) G1691A, prothrombin G20210A, and methylenetetrahydrofolate reductase (MTHFR) C677T in acutely symptomatic ambulatory patients with idiopathic pulmonary embolism (PE) has not been measured.

Methods: This prospective case–control study included patients presenting to urban emergency departments (EDs) with chest pain or shortness of breath. Cases were classified as idiopathic PE (49 patients with PE, but without overt risk factors for thrombosis). Control groups included (a) patients with nonidiopathic PE (152 patients with PE and risk factors); (b) patients in whom PE was excluded (91 patients who had PE ruled out with a structured protocol, including follow-up); and (c) patients in whom PE was not suspected (193 patients without a workup for PE, who were free of PE on follow-up). Blood DNA extracts were analyzed by PCR and restriction fragment length polymorphism analysis for the FVL, prothrombin, and MTHFR sequence variations.

Results: Either the FVL or prothrombin variant was found in 10% (95% confidence interval, 3%–22%) of patients with idiopathic PE compared with 13% (8%–20%) of nonidiopathic PE patients and 2% (5%–14%) of PE excluded patients. Patients with idiopathic PE tended to have a higher frequency of homozygous MTHFR sequence variants, but mean (SD) plasma homocysteine concentrations were not increased [15.6 (5.4) μmol/L vs 12.8 (4.6) μmol/L for homozygous, and wild-type, respectively; P = 0.40].

Conclusions: The frequency of either the FVL or prothrombin sequence variant was not increased in idiopathic PE patients compared with nonidiopathic PE patients or patients who had PE excluded. These data suggest that genotyping to detect idiopathic PE would have limited clinical utility in the urban ED setting.

Large population-based studies have found that 25% to 50% of patients with confirmed pulmonary embolism (PE)3 have no identifiable risk factors for venous thrombosis (1–4). These idiopathic cases represent a concern for emergency physicians, because patients with idiopathic PE are more difficult to identify prospectively than are patients with traditional risk factors for PE (5–8). Approximately 5%–10% of the general Caucasian population carries either the factor V (FV)4 Leiden (G1691A) or the factor II (F2; prothrombin G20210A) sequence variant, and 20%–25% of unselected patients with venous thrombosis are heterozygous for either variant (9–11). Several investigators have postulated that patients with idiopathic PE are even more likely to carry an unrecognized, inherited thrombophilia that could be detected by laboratory testing (12–14).

A patient’s pretest probability significantly affects the algorithm to diagnose or exclude PE (15–17). For any patient who self-presents to an emergency department (ED) with signs and symptoms of PE in the absence of overt risk factors for thrombosis, the clinician must con-
sider the possibility that this patient has an unrecognized thrombophilia, which might increase the patient’s pretest probability of PE. At present, no reported study has measured the frequency of thrombophilia-related genetic variants in self-reporting, acutely symptomatic patients ultimately diagnosed with idiopathic PE and compared this frequency with relevant control groups (5, 10, 12, 14).

Technologies such as real-time multiplex PCR testing allow rapid screening for multiple genomic variations. Restriction fragment length polymorphism analysis has been used to identify single-base changes responsible for thrombophilic phenotypes. Epidemiologic studies have demonstrated that patients either heterozygous or homozygous for the factor V Leiden (G1691A) or the factor II (prothrombin G20210A) sequence variants have a significantly increased risk of venous thromboembolism (4).

The significance of the methylenetetrahydrofolate reductase (MTHFR) C677T variant has less certain documentation (18), but den Heijer et al. (19) found in a recent metaanalysis that the MTHFR variant imparts a weak but statistically significant increased risk of venous thrombosis [pooled odds ratio, 1.6; 95% confidence interval (CI), 1.10–2.34]. The C677T variant encodes a thermolabile MTHFR enzyme, reducing its ability to catalyze the cobalamin-dependent remethylation of homocysteine to form methionine. The MTHFR C677T variant is believed to confer thrombogenicity only if it increases the plasma homocysteine concentration (13, 20–22).

The aim of this project was to measure the frequency of the thrombophilic genotypes (factor V Leiden, prothrombin G20210A, or MTHFR C677T with hyperhomocysteinemia) in patients with idiopathic PE (cases) compared with control patients diagnosed with PE in the presence of overt risk factors. To determine the underlying frequencies of these thrombophilia-related genetic variants in control patients without PE, we genotyped two additional groups: one group had symptoms consistent with PE, and physicians used predefined clinical protocol to rule out PE; and the other group of patients had symptoms consistent with PE, but physicians chose not to test for PE, instead testing for the alternative diagnosis, acute coronary syndrome (ACS). Suspected ACS is the most common alternative diagnosis to PE in ED patients, and physicians use the presence or absence of an alternative diagnosis to affect their assessment of pretest probability of PE (23–25).

**Materials and Methods**

**Research Setting and Participants**

This research protocol was a prospective study approved by the Carolinas Healthcare Institutional Review Board. Consecutive patients presenting with symptoms of PE, ACS, or both were eligible for the study. All research participants were adults who self-presented to the ED at Carolinas Medical Center, an urban teaching hospital in Charlotte, NC, with a census of 113,000 ED visits annually. All patients in this study had a symptom of chest pain, dyspnea, or both. Patient data were collected by the clinicians in charge of each participant’s clinical care, using real-time data entry on web-based electronic data forms, as we have described previously (26). The data-entry forms included questions asking whether the patient had any known risk factors for venous thromboembolism (26).

We studied 4 groups of patients: the case group and 3 control groups. Patients were classified as having idiopathic PE if they had PE diagnosed in the absence of the following risk factors: pregnancy or postpartum (<4 weeks) status, use of any drug containing exogenous estrogen or estrogenic drug treatment, congestive heart failure, any history of malignancy, connective tissue disease or inflammatory bowel disease, any surgery within the previous 4 weeks requiring general anesthesia, total body or limb immobilization >48 h, transatlantic air travel within the previous week, indwelling central venous catheter, previous venous thromboembolism, well-established familial history of thromboembolism, or a body mass index >40 kg/m² (27). Control groups included (a) patients with nonidiopathic PE, defined as ED patients with PE diagnosis after identification of one or more risk factor(s); (b) patients with PE excluded, defined as ED patients who had signs and symptoms that prompted a formal diagnostic evaluation for PE, but who had none of the stated risk factors; and (c) patients in whom PE was not suspected, defined as ED patients with symptoms consistent with PE (dyspnea or chest pain) but who did not have a stated risk factor and did not undergo evaluation for PE, but instead underwent formal evaluation to rule out ACS.

**Diagnostic Reference Standards**

The algorithms for diagnostic studies in all groups were performed under the supervision of a board-certified emergency physician. Diagnosis of PE in idiopathic PE cases and nonidiopathic PE patients required computed tomography angiographic evidence of a PE, read by a board-certified radiologist with specialty training in body imaging, as we have described previously (28). All PE-excluded patients were evaluated for PE according to a structured, published protocol, including 90-day follow-up, and were found to be free of PE or deep venous thrombosis (29). All PE not suspected patients were evaluated for ACS according to a published protocol that included paired biomarker measurements, provocative testing, and 45-day telephone and medical record follow-up, as described previously (30, 31). All patients in the PE not suspected group were free of both ACS and PE on 45-day follow-up.

**Sample Collection and Storage**

Blood was collected at the time of enrollment in 10-mL BD Vacutainer® plastic EDTA tubes (Becton Dickinson; cat. no. 366643). Within 5 min of collection, blood was transferred to a 15-mL polypropylene culture tube (USA Sci-
entif; cat. no. 1475-1611) and centrifuged at 2000g for 10 min at 4 °C. Fractionated plasma was aliquoted into cryovials and frozen at −70 °F.

DNA ISOLATION
DNA isolation and genotyping experiments were carried out 6–12 months after sample collection by a technician with 9 years of experience in molecular biology. The technician was blinded to the clinical data of the study samples. DNA was isolated from ≈2 mL of EDTA-treated fractionated blood by use of the QIamp DNA Blood Midi Kit (Qiagen Corporation; cat. no. 51185).

GENOTYPING PROTOCOLS
The factor V Leiden sequence variant (G1691A) was detected according to the method described by Bertina et al. (32) Briefly, a 175-bp fragment of the FV gene was amplified by use of 200 ng of genomic DNA in a total volume of 50 μL containing 50 mM KCl; 20 mM Tris-HCl (pH 8.4); 1.75 mM MgCl2; 0.05 mM each of dATP, dGTP, dCTP, and dTTP; 2.5 U of Taq Polymerase (Invitrogen; cat. no. 18038-018); and 3.3 ng of the oligonucleotide primers 5′-GCAGATCCCTGGACAGTC-3′ (underlined T indicates change to create TaqI site) and 5′-TGTATTACA-CACTGGTGCTAA-3′. The reaction mixture was cycled as follows in a DNA thermal cycler (Perkin-Elmer; Model 9600/2400): initial denaturation step of 94 °C for 5 min followed by 30 cycles of 94 °C 45 s, 54 °C for 45 s, and 72 °C for 45 s, followed by a final extension of 72 °C for 5 min. A 25-μL aliquot of the amplified fragment was then digested at 65 °C for 2 h with TaqI Restriction Enzyme (New England Biolabs; cat. no. R0149L). Digestion products were separated by electrophoresis on 3% Nusieve agarose gels and were visualized by ultraviolet light after staining with ethidium bromide. In this assay, the wild-type alleles were digested to give a 157-bp band, whereas the mutant alleles, which have lost the restriction site and will not be digested, give a band of 175 bp.

The prothrombin (G20210A) variant was detected by use of the same protocols for amplification and digestion of the region of interest as described for factor V Leiden, with the exception of the primer design and PCR product size. The primers for PCR amplification of prothrombin (G20210A) were as follows: 5′-CAATAAAAGTGACTCTCATC-3′ (underlined T indicates change to create TaqI site) and 5′-AGGTGGTGAGTTCAATGTTC-3′. These primers give a PCR product of 118 bp. Again, the wild-type allele was digested, whereas the mutant allele was not. Digestion of the wild-type PCR product gave a fragment of 98 bp, and digestion of the variant PCR product gave a fragment of 118 bp.

The C-to-T substitution at nucleotide 677 in the MTHFR gene was detected by the same methods described for factor V and prothrombin with the exception of primer design and PCR product size. The primers for MTHFR were 5′-TGAAGAGGAAGGTGTCTGCGGGA-3′ and 5′-AGGACGGTGCGGTAGAGTG-3′. PCR amplification gave a 198-bp DNA product. Subsequent overnight digestion of this product with Hinfl (New England Biolabs; cat. no. R0155L) at 37 °C yielded a 198-bp fragment for the wild-type sequence, whereas digestion of the PCR product for the variant yielded 175- and 23-bp fragments.

HOMOCYSTEINE MEASUREMENTS
To test for phenotypic manifestation of the MTHFR sequence variant, we measured homocysteine concentrations in plasma from fasted participants, using the homocysteine STE Assay (ALPCO Diagnostics; cat. no. 34-GA-HCY).

STATISTICAL ANALYSIS
We used a case–control design to allow quantitative comparison between groups, rather than assessment of risk. Accordingly, the primary analysis focused on between-group comparisons of the frequencies of sequence variations. For all variants, the proportions and their 95% CIs were calculated by the Clopper–Pearson method (Stats Direct®, Ver. 2.2.3). Homocysteine concentrations were compared by use of an unpaired t-test. For sample size estimation, we assumed that the frequency of any one variant allele for either factor V Leiden or prothrombin in patients with idiopathic PE (n = 49) would be at least 20% higher than the frequency in each of the control groups; with α = 0.05 and β = 0.20, using a two-sided test for 2 independent proportions, we thus would need at least 90 persons in each control group. An exact two-sided P value <0.017 [Bonferroni-adjusted for 3 comparisons (33)] was considered significant.

RESULTS
From January 2002 to August 2004, we enrolled 200 consecutive ambulatory patients diagnosed with PE. Of these, 49 patients (24%; 95% CI, 19%–31%) had idiopathic PE. The clinical characteristics of the PE patients (idiopathic PE and nonidiopathic PE) and symptomatic non-PE control patients (PE excluded and PE not suspected) are shown in Table 1. The mean age was similar between patients with idiopathic and nonidiopathic PE. Male sex was predominant in the idiopathic PE patient group: 65% (50%–78%) vs 36% (28%–44%) in the nonidiopathic PE patient group. The prevalence of African-American race was relatively high in all groups.

FACTOR V LEIDEN G1691A VARIANT
Among patients with idiopathic PE, 8% (95% CI, 2%–20%) were heterozygous, and none were homozygous for the factor V Leiden variant (Table 2). The frequency of the factor V Leiden variant in the PE-excluded group was 1% (0%–6%), which was statistically different from the frequency in the idiopathic PE group (95% CI for a 7% difference, 1%–18%; P = 0.03). We observed no significant difference in the frequency of the factor V Leiden variant between the idiopathic PE group and the 2 other control
groups, those with nonidiopathic PE and those in whom PE was not suspected.

**Prothrombin G20210A Variant**

Among patients with idiopathic PE, 6% (95% CI, 1%–17%) were heterozygous and none were homozygous for the prothrombin G20210A variant (Table 2). This 6% frequency was not significantly different from the frequencies of the prothrombin G20210A variant observed in any of the control groups.

**Factor V Leiden G1691A or Prothrombin G20210A**

Of the 49 patients with idiopathic PE, 5 [10% (95% CI, 3%–22%)] had at least one allele with either sequence variant, compared with 13% (5%–14%) in the nonidiopathic group, 2% (3%–8%) in the PE-excluded group, and 7% (4%–12%) in the PE not suspected group. The only comparison that reached statistical significance was between the nonidiopathic PE group and the PE-excluded group (95% CI for the 11% difference, 4%–18%; P = 0.003).

**MTHFR C677T**

Patients in the 4 patient groups had similar frequencies for either heterozygosity or homozygosity for the MTHFR variant gene at position 677 (Table 2). We observed a trend toward an increase in the frequency of homozygosity for the MTHFR variant among patients with idiopathic PE (12%; 95% CI, 5%–25%). Mean (SD) homocysteine concentrations were very similar for patients who were either homozygous [15.6 (5.4) μmol/L] or heterozygous [14.6 (5.4) μmol/L] for MTHFR C677T compared with patients with the wild-type MTHFR gene [12.8 (4.6) μmol/L; P = 0.40 for homozygous vs wild-type].

**Subanalysis of Race**

Our urban US ED patient population had a high prevalence of African Americans—a population known to infrequently carry either the factor V Leiden or prothrombin sequence variations (4). To examine possible race-related differences in genotype, we calculated the proportion of all 3 sequence variations in black patients and non-black patients in our 4 patient groups. We found only one potential difference: non-black patients with idiopathic PE carried the factor V Leiden variant more frequently than did the black patients with idiopathic PE [16% (95% CI, 5%–36%) vs 0% (0%–14%), respectively]; however, we did not formally test for statistical interaction. However, 10% (5%–18%) of non-black patients with nonidiopathic PE also carried the factor V Leiden variant. The percentages refer to patients either heterozygous or homozygous for factor V Leiden (data not shown).

**Discussion**

This study addresses the question of whether clinical decision-making for patients with signs and symptoms suggestive of PE, but without risk factors for thrombosis, could be improved if a clinician had knowledge of 3 genotypes associated with thrombophilia. We studied a prospective cohort of ED patients diagnosed with PE and focused on whether the subgroup of patients with idiopathic PE had a higher frequency of thrombophilia-related genetic variants compared with 3 clinically relevant control groups. We found that patients with idiopathic PE more frequently had one allele that carried either the factor V Leiden G1691A or prothrombin G20210A sequence compared with patients who had PE excluded. However, 90% of patients with idiopathic PE...
and 87% of patients with nonidiopathic PE were wild type for both the factor V Leiden and the prothrombin gene. Moreover, we found no difference in the frequency of the MTHFR C677T variant, and patients homozygous for this variant did not have increased fasting serum homocysteine concentrations, suggesting the absence of a phenotypic significance.

This report offers data relevant to the question of whether clinical decision-making would benefit if emergency physicians had access to real-time determination of thrombophilic genotype. Another way of viewing this project would be to ask whether the knowledge gained by genotyping would be helpful in adjusting a clinician’s pretest clinical probability of the presence of PE in patients with signs and symptoms of PE but no identifiable risk factors for PE. If we had found that the frequencies of the potent sequence variations were very high (e.g., >25%) in the group with idiopathic PE, then this hypothetical finding might point toward the need for rapid genotyping to detect idiopathic PE. The published literature suggests that persons of any race carrying either the factor V Leiden G1691A or prothrombin G20210A variant are at significantly increased risk for venous thromboembolism; we therefore believed it was important to compare the frequency of either sequence variation among groups (4, 34–37). We found that 5 of 49 (10%; 95% CI, 3%–22%) patients with idiopathic PE had either the factor V Leiden or prothrombin sequence variant. This proportion was not significantly higher than the proportion in the PE-excluded patient group [2 of 91; 2% (3%–8%)].

We included 3 control groups to enhance the clinical relevance of this report. In real practice, clinicians often face the dilemma of when to order expensive, time-consuming, and potentially invasive imaging procedures for PE in a patient with chest pain or dyspnea but with no overt risk factors for PE. At the time and place of this scenario, the clinician must consider multiple potential threats to life in the differential diagnosis, including PE and ACS. Accordingly, we believed it was imperative to include the 2 additional control groups who, in prospect, “looked clinically similar” to the patients with idiopathic

### Table 2. Prevalence of factor V Leiden, prothrombin, and MTHFR sequence variations.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Idiopathic PE (n = 49)</th>
<th>Nonidiopathic PE (n = 152)</th>
<th>PE excluded (n = 91)</th>
<th>PE not suspected (n = 193)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor V Leiden</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>45 (92%) (90–98%)</td>
<td>140 (92%) (87–96%)</td>
<td>90 (99%) (94–100%)</td>
<td>182 (94%) (90–97%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>4 (8%) (2–20%)</td>
<td>11 (7%) (4–13%)</td>
<td>1 (1%) (0–6%)</td>
<td>10 (5%) (3–9%)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>0 (0%) (0–7%)</td>
<td>0.7 (0.02–4%)</td>
<td>0 (0–4%)</td>
<td>0.5 (0–3%)</td>
</tr>
<tr>
<td><strong>Prothrombin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>46 (94%) (83–99%)</td>
<td>143 (94%) (89–97%)</td>
<td>90 (99%) (94–100%)</td>
<td>188 (97%) (94–99%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>3 (6%) (1–17%)</td>
<td>8 (5%) (2–10%)</td>
<td>1 (1%) (0–6%)</td>
<td>3 (2%) (0.3–5%)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>0 (0%) (0–7%)</td>
<td>0.7 (0.02–4%)</td>
<td>0 (0–4%)</td>
<td>0 (0–1%)</td>
</tr>
<tr>
<td><strong>MTHFR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>30 (61%) (46–75%)</td>
<td>90 (59%) (51–67%)</td>
<td>51 (56%) (45–66%)</td>
<td>130 (67%) (60–74%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>13 (27%) (15–41%)</td>
<td>54 (36%) (28–44%)</td>
<td>35 (39%) (28–49%)</td>
<td>51 (26%) (20–33%)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>6 (12%) (5–25%)</td>
<td>8 (5%) (2–10%)</td>
<td>5 (6%) (2–12%)</td>
<td>12 (6%) (3–11%)</td>
</tr>
</tbody>
</table>

*P = 0.03 vs PE-excluded controls.*
If the prevalence of any of the 3 genetic variants was very low in these control groups compared with the patients with idiopathic PE, this hypothetical observation might have provided some support for the use of rapid genotyping. We found that 2% (95% CI, 0%–7%) of PE-excluded patients (those who underwent testing for PE but did not have PE) and 7% (4%–12%) of PE not suspected patients had either the factor V Leiden or the prothrombin variant. We interpret these data to indicate that genotyping would be very unlikely to prospectively help the clinician decide which patient with signs and symptoms of PE, but no overt risk factors, should be evaluated by a thorough pulmonary vascular imaging protocol.

Several limitations might affect the external validity of this study. If this study was repeated in an ED with a lower percentage of African Americans, the results might be different. Another limitation to this study is the apparently low number of patients with idiopathic PE (n = 49) drawn from a prospectively enrolled cohort of 200 patients with diagnosed PE. As a result, the CIs for the proportions of genotypes in the idiopathic PE group were relatively wide. Nonetheless, we believe this to be the largest published study of genotyping for prospectively studied, self-reporting, symptomatic patients diagnosed with idiopathic PE. If a subsequent study were to find a prevalence of either the factor V Leiden or the prothrombin variant at the top of our 95% CI (22%), this finding might not strongly support routine genotyping to detect idiopathic PE. It might also be argued that other thrombophilic genotypes would be more frequent and more important. We believe, however, that the 3 genotypes that we reported had the widest recognition in 2005.

In summary, 10% (95% CI, 3%–22%) of symptomatic, self-reporting urban ED patients with idiopathic PE had either factor V Leiden (G1691A) or prothrombin G20210A compared with 13% (5%–14%) of patients with nonidiopathic PE. The frequency of thrombophilic genotypes was not increased in patients with idiopathic PE compared with patients who had PE excluded. We conclude that testing for thrombophilic genotype would seldom aid in identifying patients at risk for idiopathic PE in the ED setting.

This work was funded by National Institutes of Health National Heart, Lung, and Blood Institute Grant R01 HL074384 (to J.A. Kline) and the Emergency Medicine Foundation’s Rigg’s Family Heart Policy Award (to J.A. Kline).

References
22. de Moerloose P, Bounnameaux HR, Mannucci PM. Screening tests


