The ACE D/D genotype is protective against the development of idiopathic deep vein thrombosis and pulmonary embolism

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Summary
The deletion/deletion (D/D) genotype of the angiotensin converting enzyme (ACE) has been purported to be a risk for post-operative thrombosis. This D/D genotype has not been evaluated as a risk factor for idiopathic venous thromboembolism (VTE).

The primary objective of the present study was to determine whether the D/D genotype of ACE is independently associated with the occurrence of idiopathic venous thromboembolic disease.

We prospectively enrolled consecutive patients with at least one objectively confirmed idiopathic VTE. Friends of cases were recruited as controls and matched to cases by sex, ethnicity, and age. Patients were tested for the ACE I/D polymorphism in addition to factor V Leiden, prothrombin G20210A, and factor VIII levels.

Three hundred cases and 300 controls were enrolled; 97% were Caucasian. There were 148 females and 152 males in each group with a mean age of 56.21 years (SD=15.33). The ACE D/D genotype was present in 25.3% of cases and 32.4% of controls for an adjusted odds ratio of 0.66 (95% CI = 0.433 to 0.997).

We can conclude that the ACE D/D genotype is protective against idiopathic venous thromboembolism.

Keywords
ACE, angiotensin converting enzyme, venous thromboembolism, deep vein thrombosis, pulmonary embolism

Introduction
In the last decade a number of thrombophilias have been identified; several are due to genetic abnormalities. These discoveries have helped to explain why some individuals and families are more susceptible to venous thromboembolism (VTE).

However, between 35-52% of patients with idiopathic VTE have no known genotypic/phenotypic cause for the disease, suggesting that there are additional thrombophilias that are yet to be recognized (1). Levels of angiotensin converting enzyme (ACE) are reported to be affected by genotype, and either the genotype or the enzyme levels appear to be possible, but as yet unproven,
thrombophilia candidates. The gene producing ACE contains a polymorphism consisting of an insertion (I) or deletion (D) of a 287 base pair fragment of intron 16 of the ACE gene resulting in three genotypes: D/D and I/I homozygotes and I/D heterozygotes. This polymorphism has been shown to account for over 50% of the inter-individual variation in plasma ACE concentrations. In one report patients with the D/D genotype had plasma ACE levels about twice those in individuals with the I/I genotype (2).

Laboratory studies with rat models have suggested an association between ACE levels and VTE (3, 4). Several case control studies investigating the association of ACE with VTE have been reported in abstract form and five studies have been published but no conclusions have been reached. The published studies have had conflicting results, reporting odds ratios for VTE with the deletion/deletion (D/D) genotype between 0.64 and 11.7 (Table 1) (5-9). Design limitations present in these studies and many other previous case control studies may account for differing results. Limitations include potential referral or selection bias, small or heterogeneous samples, use of inappropriate controls, and inadequate matching. Differences across studies in subject characteristics relevant to thrombosis risk, including race, age, gender, and the presence of risk factors for VTE, may also be responsible for conflicting results. It is quite clear that patients with VTE as a consequence of transient risk factors or cancer differ in risk for initial and recurrent thrombosis (10). A small study with patients post total hip arthroplasty reported an odds ratio of 11.7 for those with the D/D genotype (8). Authors of the largest ACE study postulated that the D/D genotype may be a risk factor associated exclusively with VTE secondary to hip replacement (9). Specifically, the altered vascular tone and blood flow associated with higher circulating ACE levels may compound the effects of reduced venous blood flow with anesthesia and surgery. To date, there have been no studies that have investigated the ACE D/D genotype exclusively in patients with idiopathic VTE.

The primary objective of the present study was to determine whether the D/D genotype of ACE is independently associated with the occurrence of idiopathic VTE. Secondary objectives were to verify the previously determined increased risk of idiopathic VTE in patients with factor V Leiden and the prothrombin gene defect G20210A.

### Methods

#### Subjects

This case-control study was conducted at the Ottawa Hospital with approval from the institutional research ethics board. Cases were recruited from our Thrombosis Clinic, which serves as a referral base for a community of approximately 700,000 people. Consecutive patients with at least one objectively confirmed idiopathic DVT or PE and who had completed three months of follow-up were eligible for inclusion. Patients were excluded if they had a malignant disorder.

Friends of cases were recruited as control subjects. Friend controls have been used successfully in past genetic studies of VTE and allow for accurate age and sex matching (11-14). Controls were matched to cases by sex, ethnicity, and age (using increments of five years). All potential controls were interviewed with a previously validated questionnaire (15) to exclude venous thromboembolic disease and to ensure they were not misclassified cases. Controls were excluded if they had used warfarin, or other oral anticoagulants, in the last three months, had prior VTE or malignant disease. Trained staff

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**Table 1**: Characteristics of published studies examining the association between the ACE D/D genotype and venous thromboembolism (VTE).

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample Size</th>
<th>Proportion of idiopathic and secondary VTE</th>
<th>Race</th>
<th>Age (years)</th>
<th>Gender (female)</th>
<th>Type of Controls</th>
<th>Odds Ratio for VTE (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wells et al.</td>
<td>300 cases</td>
<td>All idiopathic</td>
<td>Caucasian 97%</td>
<td>Mean 56</td>
<td>49%</td>
<td>friend controls with no history of VTE matched by age, sex, ethnicity</td>
<td>0.66 (0.433-0.997)</td>
</tr>
<tr>
<td>Jackson et al.</td>
<td>517 cases</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Median 41</td>
<td>69%</td>
<td>anonymous blood donors, unmatched</td>
<td>0.97 (0.81-1.16)</td>
</tr>
<tr>
<td>Dilley et al.</td>
<td>93 cases</td>
<td>Not reported</td>
<td>African-American 100%</td>
<td>Mean 55</td>
<td>58%</td>
<td>attendees of a clinic lab for routine blood tests matched by age, sex, race</td>
<td>1.5 (0.9-2.6)</td>
</tr>
<tr>
<td>Philipp et al.</td>
<td>30 cases</td>
<td>All secondary to total hip arthroplasty</td>
<td>Caucasian 93%</td>
<td>Mean 69</td>
<td>33%</td>
<td>patients undergoing total hip arthroplasty matched by age, sex, and date of operation</td>
<td>11.7 (2.3-84.5)</td>
</tr>
<tr>
<td>Lu et al.</td>
<td>72 cases</td>
<td>*</td>
<td>Chinese 100%</td>
<td>*</td>
<td>*</td>
<td>Healthy controls matched by sex and age</td>
<td>2.5 (*)</td>
</tr>
<tr>
<td>Gonzalez</td>
<td>148 cases</td>
<td>Not reported</td>
<td>Caucasian 100%</td>
<td>Mean 45</td>
<td>46%</td>
<td>Hospital staff or blood bank donors of same ethnicity, unmatched</td>
<td>0.64 (0.42-0.99)</td>
</tr>
<tr>
<td>Ordonez et al.</td>
<td>72 controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*information not available in English abstract; original article in Chinese journal not obtained*
obtained a complete clinical summary from all patients with emphasis on personal history of environmental risk factors for venous thromboembolism (smoking, BMI, surgery, immobilization, pregnancy, hospitalization, postpartum, trauma, varicose veins, and cancer; and use of oral contraceptives, ACE inhibitors, hormone replacement therapy, antiplatelet agents). Data were also collected on the presence of hyperlipidemia, diabetes, hypertension, coronary artery disease, peripheral vascular disease, stroke, personal history of thrombosis, and family history of thrombosis.

Blood collection and laboratory analysis

In addition to the ACE I/D polymorphism, the thrombophilias tested included factor V Leiden, prothrombin G20210A, and factor VIII. Elevated factor VIII levels were repeated to rule out falsely abnormal results. Cases had their blood samples drawn at least three months after the DVT or PE. Blood was collected into vacuum tubes that contained 0.129 M trisodium citrate as an anticoagulant. Samples were centrifuged at 3000 g for 20 minutes at 4°C to obtain platelet poor plasma, and then they were aliquoted into plastic tubes and frozen and stored at -70°C. Assays were performed in batches.

After thawing in a 37°C water bath for five minutes factor VIII assays were performed using the PTT based Diagnostica Stago (Abbott Diagnostics Canada Limited, Mississauga) on the STA compact™ automated coagulation factor analyzer. Quality control measures included laboratory enrollment in the Ontario LPTP licensing program and an industry proficiency quality assurance program. Suitable controls and calibrators were run alongside research specimens as per the recommendations of the manufacturer.

Standard protocols were used to extract DNA from peripheral blood leukocytes. All mutation analysis was performed on DNA isolated from leukocytes. Genotyping for factor V Leiden (1691A→G) and prothrombin (20210G→A) was performed using established methods (16). In brief, factor V 1691A→G is detected by Mnl I digestion of an amplicon encompassing nucleotide 1691. The prothrombin 20210G→A transition is detected through the use of a mutagenic primer that introduces a Hind III restriction site to amplicons containing the 20210A allele.

The ACE insertion or deletion was detected using agarose gel separation of PCR amplified DNA from intron 16. PCR products at 319 bp indicate the deletion genotype while an amplicon at 597 bp indicates the insertion genotype (17). Subjects genotyped for D/D were reamplified with primers specific for the insertion genotype. A 335 bp PCR product indicates the presence of the insertion genotype (18). If no product was amplified in this reaction then the patient was a true D/D genotype.

Statistical analysis

We calculated power via Monte Carlo simulation. Actual logistic regression modeling was carried out on simulated data and the percent of time statistical significance occurs was calculated. We did conditional logistic regression just for the first set of rates and used unconditional logistic regression to investigate a range of underlying case/control rates. We simulated 300 cases and controls, matched on five year age ranges, and fit main effects and one interaction term (ACE D/D * Factor V Leiden) using conditional logistic regression. Three hundred cases and controls were determined to be adequate for the interaction and clearly adequate for the main effect, which is the primary objective of the study.

Logistic regression analysis was used for the primary analysis of the association between the ACE D/D genotype and idiopathic VTE. Factor V Leiden, factor VIII levels (above and below 267 IU/dl), the prothrombin gene mutation G20210A, body mass index (BMI), smoking, personal history of VTE and family history of VTE were initially entered stepwise into the regression in order to find a preliminary model. We also performed “matched pair” logistic regression conditioned on age, gender and ethnicity, and adjusting for the variables previously listed. We did not adjust for anticoagulants, antiplatelet drugs, ACE inhibitors, hormone replacement therapy, and oral contraceptive use given that drug recall is often faulty and the index venous thromboembolic event was not necessarily recent. To determine which variables would be included in our final logistic regression model without stepwise selection we used statistical analysis, correlation of dependent and independent variables and expert clinical opinion. The variables that remained in the final multivariate model were factor V Leiden, factor VIII, prothrombin G20210A, BMI, and smoking. This final model was used for our secondary analyses to determine the odds ratio for development of VTE for factor V Leiden, and the prothrombin gene mutation G20210A.

We also conducted exploratory subgroup analysis for males and females, for cases with a first VTE and recurrent VTE, and separately for cases with DVT alone or PE, appreciating that these analyses would be underpowered. Prevalence odds ratios (OR) and 95% confidence intervals (CIs) were determined according to the logit distribution.

Results

Three hundred cases and 300 controls were enrolled and included in the unadjusted analysis. Both plasma and DNA samples for all required assays were available for 290 cases and 290 controls so only these subjects were included in the matched and adjusted odds ratio calculations. There were 148 females and 152 males in each group with a mean age of 56.21 years (SD=15.33). In both groups 97% of subjects were Caucasian. One hundred and ninety cases had one VTE, 65 had two, and 45
had more than two prior VTE. There were 115 cases that had
PE, with or without DVT. All thrombotic events had been con-
ferred with objective diagnostic methods. At the time of the
study, 233 cases were on warfarin, and 37 cases and 32 controls
were on ACE inhibitors.

The prevalence of the thrombophilias in cases and controls,
and the odds ratio and the 95% confidence intervals for the pro-
thrombosis G20210A gene defect and for factor V Leiden are
listed in Table 2. The ACE D/D genotype was present in 26.2%
of cases and 32.4% of controls, the I/D genotype in 54.5% of
cases and 49.7% of controls, and the I/I genotype in 20.1% of
cases and 17.9% of controls. The distribution of genotypes in
cases and controls was consistent with Hardy-Weinberg equilib-
rium. The adjusted odds ratio for VTE with the D/D genotype
was 0.66 (95% CI = 0.432-0.997) and was similar in other
models regardless of matching and adjustment. Testing the
dominant hypothesis of I/I vs. D/D and I/D gave an odds ratio
of 1.15 (95% CI = 0.76-1.75). The prothrombin G20210A gene
defect was present in 8.7% of cases and 2.0% of controls, and
the factor V Leiden gene was present in 23.8% of cases and
49.7% of controls, and the I/I genotype in 20.1% of
cases and 17.9% of controls. The distribution of genotypes in
cases and controls was consistent with Hardy-Weinberg equilib-
rium. The adjusted odds ratio for VTE with the D/D genotype
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dominant hypothesis of I/I vs. D/D and I/D gave an odds ratio
of 1.15 (95% CI = 0.76-1.75). The prothrombin G20210A gene
defect was present in 8.7% of cases and 2.0% of controls, and
the factor V Leiden gene was present in 23.8% of cases and
4.6% of controls. The Hosmer and Lemeshow goodness-of-fit
test indicated that our adjusted model explained 75% of the
variation in the data.

In the exploratory subgroup analyses the odds ratio for VTE
with the D/D genotype was 0.822 (95% CI = 0.451-1.501) for
females and 0.543 (95% CI = 0.303-0.973) for males. For those
cases with a first VTE the odds ratio was 0.756 (95% CI =
0.476-1.200) and for recurrent VTE it was 0.562 (95% CI =
0.306-1.033). When cases with PE were compared to controls
the odds ratio was 0.761 (95% CI = 0.472 - 1.227) and for those
with DVT alone the odds ratio was 0.624 (95% CI = 0.411 -
0.948).

Discussion

This investigation is the first case-control study to look exclu-
sively at consecutive, unselected patients with idiopathic VTE
and to carefully match cases and controls. The adjusted odds
ratio for VTE was 0.66, indicating that the ACE D/D genotype
protects against idiopathic VTE. The odds ratio was similar
regardless of matching and adjustment. This finding furthers the
understanding of the ACE D/D genotype and its role in throm-
bosis and supports the hypothesis that the ACE D/D genotype
protects against the development of venous thrombosis. We
performed several subgroup analyses and in all cases the odds
ratios were consistent with our primary outcome. However,
there were some interesting trends which are discussed below.

There are several potential limitations of published studies
examining genetic disorders as causes of VTE that may lead to
inaccurate results. We addressed these in the design of our
study. The first potential limitation of studies to date is the
broad spectrum of patients typically enrolled, including all
available patients regardless if the VTE was idiopathic or sec-
ondary to transient risk factors or malignancy. We enrolled only
patients with idiopathic VTE. It is possible that the presence of
environmental risk factors alters the association between genetic
abnormalities and VTE (12;19). Hence it may not be appro-
priate to mix idiopathic and secondary thrombosis patients since
thrombotic risk may be very different in these populations. It is
quite clear that the natural history of VTE depends on whether
the thrombosis was secondary or idiopathic. Patients with
idiopathic VTE have a high risk of recurrent events while those
with VTE secondary to transient risk factors do not (20). In
patients exposed to transient VTE risk factors, it is possible that
the association between candidate thrombophilia and the occur-
rence of VTE may be weakened or altered in an opposite direc-
tion. In addition our study indicated a trend for the ACE D/D
genotype being more protective for recurrent VTE than in
patients with one VTE, hence it may be important to separate
these types of patients in future studies.

The five published studies that have specifically evaluated
VTE risk with the ACE D/D genotype all have limitations. Phillip
colleagues enrolled a small homogeneous sample of
patients post total hip arthroscopy. They reported an OR of 11.7
with a wide confidence interval (2.3-84.5) indicating this OR is
imprecise (8). This study may not be comparable to ours since
all subjects were screened for DVT with routine bilateral ultras
ound and it was not clear what proportion of thrombi were
actually symptomatic or clinically relevant. Furthermore, even
though this study suggested a relationship between the D/D ge
notype and secondary thrombosis, this does not necessarily
imply the D/D genotype high ACE levels will increase the
risk for idiopathic thrombosis. For example, even though factor
V Leiden is clearly associated with idiopathic DVT, there are data

<table>
<thead>
<tr>
<th>Cases</th>
<th>ACE D/D Genotype</th>
<th>Factor V Leiden</th>
<th>Prothrombin G20210A</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.3%</td>
<td>23.6%</td>
<td>8.7%</td>
<td></td>
</tr>
<tr>
<td>32.4%</td>
<td>4.6%</td>
<td>2.0%</td>
<td></td>
</tr>
<tr>
<td>0.66 (0.433-0.997)</td>
<td>8.33 (4.37-15.88)</td>
<td>3.60 (1.37-9.46)</td>
<td></td>
</tr>
<tr>
<td>-0.211 (0.107)</td>
<td>2.060 (0.165)</td>
<td>0.640 (0.246)</td>
<td></td>
</tr>
<tr>
<td>0.048</td>
<td>&lt;0.0001</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

* adjusted for factor V Leiden, prothrombin G20210A, factor VIII, ACE genotype, BMI, smoking

Table 2: Prevalence of tested thrombo-
philias and adjusted* Odds Ratios.
to suggest factor V Leiden is not a risk factor for post-operative thrombosis (19). Influences on vascular tone and endothelial cell damage are much more important in the post-operative setting. General anesthetics are known to cause significant vasodilation and endothelial cell damage (21) and of course these vasodilatory effects do not occur in ambulatory patients. The endothelial damage induced by orthopedic surgery may result in more complex genetic interactions and the finding of an association with the D/D genotype may be spurious. In patients exposed to transient risk factors for VTE it is possible that an association between a candidate thrombophilia, and the development of thrombosis, could be weakened or altered to an opposite effect. Therefore, the possibility that the ACE D/D genotype may contribute to thrombotic risk through gene-environment interactions cannot be excluded by our study but this would be unlikely.

The three larger studies did not report what proportion of enrolled patients had secondary or idiopathic VTE (5, 6, 9) and a fourth study was published in Chinese and is only available to us in abstract form (7). Our findings were more compatible with these studies, two of which reported an odds ratio less than one, suggesting the D/D genotype may be protective. The largest study by Jackson and colleagues enrolled cases with a median age of 41 years and the age of the anonymous control subjects was not known (9). Enrolling young patients is not uncommon in studies examining the association between genetic abnormalities and VTE (22-24) however, it may indicate that there is referral bias in the selection of cases with VTE. Other studies of unselected patients with idiopathic VTE have enrolled patients with a mean age of 60 (25, 26). It is well accepted that the frequency of VTE increases with age. Ridker and his colleagues found that the factor V Leiden mutation was a more common cause of thrombosis in older patients than younger ones, thus demonstrating that there is an interaction between age and a genetic risk factor (27).

We matched by age, ethnicity, and gender to control for their effects. Most of our patients were Caucasian so it is not clear if our results apply to other racial groups. In a subgroup analysis, Dilley and colleagues demonstrated an increased risk of VTE for men with the ACE D/D genotype (p=0.01) but the population was limited to African-Americans (5). Although our exploratory subgroup analysis was underpowered, it showed trends contrary to these findings. This may represent a difference between Caucasians and African-Americans since genetic polymorphisms vary by ethnicity, as will gene-gene interactions and other unknown risk factors when distinct races and ethnic groups are studied. Our study was similar to Dilley’s with respect to the importance of gender because women with the ACE D/D genotype had an odds ratio different than men, and in Dilley’s study the difference between the sex-specific odds ratios approached statistical significance. Philipp and colleagues explored gender as a potential confounder in a multivariate model, but it did not change the association between genotype and thrombosis (8). Jackson and colleagues used anonymous blood donors as their controls and were unable to investigate whether gender was a potential confounder; moreover, 69% of enrolled cases were female (9).

Rosendaal and others now believe that VTE is a “multicausal disease” which is to say that multiple risk factors must be present for VTE to develop (12). Although Rosendaal proposes gene-gene interactions, there is very little literature to date on the incidence of co-inheritance of the thrombophilias. The presence of more than one genetic defect or homozygous inheritance of gene defects appears to increase thrombotic risk at least in some studies with small numbers of patients (28-30). More information on whether multiple gene inheritance results in a synergistically increased risk is needed. Regardless, it is clear that any study assessing the possibility of a new genetic defect must measure other gene defects in addition to controlling for other risk factors (so-called gene-environment interactions) that predispose to thrombosis. Data on co-inheritance is important to accurately quantify risk. While gene-gene interactions are important to consider when designing case-control studies, we found that adjusting for factor V Leiden, prothrombin, and factor VIII levels had little effect on the odds ratio associated with the ACE D/D genotype. This suggests that gene-gene interactions between the ACE D/D genotype and the other three thrombophilias do not play an important role in thrombosis risk. The odds ratios for factor V Leiden and the prothrombin G20210A defect were similar to those reported by others, confirming their pathogenic role in idiopathic VTE.

The preferred method for selecting controls is somewhat controversial. It is clear that controls need to be matched closely on age as age is a consistent risk factor for VTE and may confound an association between a thrombophilia and VTE. We matched by specific age categories of 30-34, 35-39, 40-44, etc. as described for the design of case-control studies by Rothman and Greenland (31). Clinic controls and friend controls offer different advantages. Clinic controls are almost definitely from the same source population as the cases by the fact that they have been referred to the same clinic supposedly for the same reasons, but there is always the possibility they differ from the cases in a way that confounds. Friend controls on the other hand have been successfully used in past genetic studies of VTE (11-14). This allows more accurate age and sex matching. A potential drawback of friend controls for evaluating environmental issues is that friends tend to have similar exposure status as the cases (e.g., smoking, exercise, alcohol use, interests, etc.) but this is an advantage for genetic studies when it is desirable to match for environmental exposures. Despite enrolling a sizable sample of 517 cases and 478 controls, Jackson and colleagues used anonymous blood donors as controls and no matching was possible (9). This design risks the chance that control subjects may have experienced a venous thromboembolic event and thus
were misclassified. Alternatively, in the study by Dilley and colleagues, it is possible that some cases were misclassified because 11% of the cases did not have the diagnosis of VTE confirmed by objective tests (5).

In conclusion, our data strongly suggest that the ACE D/D genotype protects against idiopathic VTE. Future studies should investigate possible gender differences and the role of the ACE D/D genotype in recurrent thrombosis and the mechanism by which the D/D genotype is protective. We did not measure plasma ACE levels so it is unclear if ACE levels are involved in the protective effect. Furthermore, it will also be important to determine if there is an association between the use of ACE inhibitors and the development of idiopathic VTE as this may have therapeutic and prophylactic implications.

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We wish to acknowledge Ferne Shirley and Marisa Freedman for their assistance with laboratory analysis.

References