Targeted Gene Sequencing Identifies Variants in the Protein C and Endothelial Protein C Receptor Genes in Patients With Unprovoked Venous Thromboembolism

Cynthia Wu,* Dhruva J. Dwivedi,* Laura Pepler, Zakhar Lysov, John Waye, Jim Julian, Karl Desch, David Ginsburg, Jeffrey I. Weitz, Clive Kearon,† Patricia C. Liaw†

Objective—The interaction of protein C (PC) with the endothelial PC receptor (EPCR) enhances activated PC generation. We performed targeted gene sequencing of the PC gene (PROC) and EPCR genes (PROCR) in patients with unprovoked venous thromboembolism (VTE) to determine whether mutations that impair PC–EPCR interactions are associated with an increased risk of VTE.

Approach and Results—We sequenced exon 3 of PROC and exons 2 and 3 of PROCR (the exons that encode the protein–protein binding domains of PC and EPCR) in 653 patients with unprovoked VTE and in 627 healthy controls. Five single nucleotide variants, each in individual patients, were identified that result in abnormal PC (Arg9Cys, Val34Met, and Arg1Cys) or abnormal EPCR proteins (Arg96Cys and Val170Leu). We did not detect any nonsynonymous coding variants in the controls. When the PC variants were expressed in human embryonic kidney 293 cells, all exhibited decreased synthesis, and 2 of the variants had reduced capacity for activated PC generation. When expressed on the surface of human embryonic kidney 293 cells, the EPCR variants showed reduced affinity for fluorescently labeled PC. In addition, the previously reported EPCR A3 haplotype, which promotes cellular shedding of EPCR, is over-represented in the patient group (P=0.001).

Conclusions—This is the first targeted DNA sequencing analysis of PROC and PROCR in a large group of patients with unprovoked VTE. Our data suggest that mutations that impair PC–EPCR interactions may be associated with an increased risk of VTE. (Arterioscler Thromb Vasc Biol. 2013;33:2674-2681.)

Key Words: endothelial cell protein C receptor ■ genes ■ polymorphism, genetic ■ protein C ■ venous thromboembolism

The protein C (PC) pathway plays a major role in inhibiting blood coagulation. PC is activated on the surface of vascular endothelial cells by the thrombin–thrombomodulin (TM) complex. The endothelial PC receptor (EPCR), a receptor which binds circulating PC and presents it to the thrombin–TM complex, enhances PC activation by ≥8-fold in vitro and by ≥20-fold in vivo. Activated PC (APC), in conjunction with its cofactor protein S, degrades coagulation cofactors Va and VIIIa, thereby attenuating thrombin generation. Hereditary thrombophilia is identified in about one third of patients with unprovoked venous thromboembolism (VTE). Most hereditary thrombophilic defects are related to decreased levels of anticoagulant proteins (eg, deficiencies of PC, protein S, or antithrombin) or gain of function in procoagulant factors (eg, increased levels of prothrombin, factor VIII, or other coagulation factors). Family history is a strong risk factor for VTE, suggesting that many more patients with unprovoked VTE have as yet unidentified genetic abnormalities that predispose them to thrombosis. Identification of these abnormalities is important because it enables more comprehensive counseling of patients and family members, including more vigilant use of VTE prophylaxis in high-risk situations (eg, prolonged immobility perioperatively) and avoidance of estrogen therapy. The presence of thrombophilia, if it were a strong predictor of recurrent VTE, could also influence the duration of anticoagulation therapy. PC deficiency is generally subdivided into 2 types. Type I deficiencies are characterized by decreases in PC antigen,
whereas type II deficiencies are characterized by abnormal biological activity. Abnormal PC activity can be assessed by amidolytic assays (which can detect abnormalities in the serine protease domain of PC) and by coagulation tests, such as those based on the activated partial thromboplastin time. In both the amidolytic and the clotting assays, the PC is commonly converted to APC by protac, an activator found in snake venom. In vivo, however, the conversion of PC to APC is monly converted to APC by protac, an activator found in snake venom. In vivo, however, the conversion of PC to APC is accelerated by the binding of PC to EPCR on the vascular endothelial cell surface. Thus, genetic mutations that impair PC–EPCR interactions would escape detection with currently available diagnostic assays.

PC contains a Gla domain (which contains 9 γ-carboxylated glutamic acid residues), a connecting region, 2 EGF-like domains, an activation peptide, and the serine protease domain. According to the crystal structure, the majority of PC residues contributing to EPCR binding are located on the α-loop of the Gla domain (which is encoded by exon 3 of the PC gene). EPCR consists of an α1 and α2 domain, which forms a ligand-binding groove composed of 2 antiparallel α-helices that sit on an 8-stranded β-sheet platform. Mutagenesis studies of EPCR and the EPCR crystal structure have shown that the PC-binding domain is located at the distal end of the 2 α-helical segments (encoded by exons 2 and 3 of the EPCR gene).

We hypothesized that mutations that impair PC–EPCR interactions may be associated with an increased risk of VTE. To explore this possibility, we sequenced the PC gene (PROC and EPCR genes) in 657 patients with unprovoked VTE (from the Extended Low-intensity Anticoagulation for Thrombo-embolism [ELATE] trial that compared low-intensity and conventional-intensity anticoagulation with warfarin for the prevention of recurrent VTE) and in 627 controls with no known history of VTE. We focused our DNA sequencing on the following regions: (1) exon 3 of PROC (which encodes for the Gla domain of PC, the region of PC that binds to EPCR), (2) exons 2 and 3 of PROCR (which encode the PC-binding domain of EPCR), and (3) exon 4 of PROCR (which contains the previously reported EPCR A3 haplotype polymorphism site). The EPCR A3 haplotype is associated with reduced EPCR function because of increased endothelial shedding of EPCR. This study is the first targeted DNA gene sequencing analyses of the PROC and PROCR genes in a large group of patients with unprovoked VTE.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
A total of 653 warfarin-treated patients with unprovoked VTE were included in this study (mean age, 57±15 years; 45% women). VTE had occurred once in 27%, twice in 51%, more than twice in 22%, and the most recent episode of VTE was proximal deep vein thrombosis only in 66% and pulmonary embolism (with or without deep vein thrombosis) in 34.14 The 627 controls who never had VTE (described in the Materials and Methods in the online-only Data Supplement) had a mean age of 42±9 years, and 49% were women.

To analyze the EPCR-binding domain of PC, we sequenced a 650-bp fragment of PROC that codes for the Gla domain of PC. A list of all single nucleotide variants (SNVs) or single nucleotide polymorphisms found in this study are summarized in Table 1 according to the Human Genome Variation Society nomenclature. We found 6 SNVs in the 650-bp fragment encompassing the coding region of the Gla domain of PC (Table 2). The first was a C to T transition at nucleotide (nt) 2896, which results in substitution of Arg with Cys at residue −1 in the Gla domain of PC (amino acids are numbered from the N terminus of the mature secreted protein). The second SNV was a C to T transition at nt 2923, which results in an Arg9Cys substitution in the Gla domain of PC. The third SNV was a G to A transition at nt 2998, which results in a Val34Met substitution in the Gla domain of PC. The remaining SNVs were located in intron 2 (C2633G and C2730T). None of these SNVs was present in the controls. We also found common single nucleotide polymorphisms in intron 3 of the PC gene in both the patients and the controls.

Using stable transfections, we expressed wild-type human PC (WT PC) and the 3 PC variants (PCArg-1Cys, PCArg9Cys, and PCVal34Met) in human embryonic kidney (HEK) 293 cells. All cell lines contained similar levels of PC mRNA as assessed by the measurement of real-time polymerase chain reaction (PCR); the PCArg-1Cys, PCArg9Cys, and PCVal34Met cell lines contained 57%, 172%, and 134%, respectively, of mRNA relative to the WT PC cell line. The cell lysates and conditioned medium were then subjected to Western blot analysis. As shown in Figure 1, WT PC was detected in the cell lysate and in the conditioned medium. The 2 PC bands in the conditioned medium are consistent with the α and β forms of PC, which differ in glycosylation state at Asn329. In contrast, PCArg-1Cys, PCArg9Cys, and PCVal34Met were detected at lower levels in the cell lysates and in the conditioned medium.

To determine whether these PROC mutations result in a type I PC deficiency, we measured PC antigen levels in 21 of the patients with VTE (3 patients had mutations in the Gla domain of the PROC gene and 18 did not). We also measured PC antigen levels in 6 healthy volunteers. As shown in Figure 2, the mean PC antigen level in the VTE patients without the PROC Gla domain mutations (3.61±0.81 µg/mL) was similar to that in the healthy volunteers (3.88±0.19 µg/mL; P=0.19). In contrast, the mean PC antigen level in the VTE patients with the PROC Gla domain mutations (2.52±0.15 µg/mL) was lower than that in healthy volunteers (P<0.001) and in the VTE patients without the PROC mutations (P=0.037). The PC antigen levels in the patients harboring the Val34Met, Arg9Cys, and Arg-1Cys mutations were 66%, 66%, and 58% of normal
levels, respectively, suggesting that these mutations result in a heterozygous type 1 deficiency.

Given that the PROC mutations are located in the Gla domain of PC, it is also possible that these mutations result in a heterozygous type IIb deficiency because of impaired ability to bind to EPCR (ie. normal amidolytic activity but reduced APC anticoagulant activity). To explore this possibility, the PC variants were purified from the conditioned medium of the stable cell lines and subjected to PC activation assays. As shown in Figure 3, the activation rate of WT PC is ≈4-fold higher on HEK293 cells expressing EPCR and TM (gray bars) compared with that observed on HEK293 cells (white bars). Preincubation of the cells with an anti-EPCR antibody that blocks PC–EPCR interactions (JRK 1535; 500 nmol/L) inhibits the activation rate of WT PC on HEK293/EPCR/TM cells. The activation rate of PCArg9Cys is similar to that of WT PC, whereas PCArg-1Cys and PCVal34Met have impaired capacity to generate APC. Thus, patients carrying the PCArg-1Cys and PCVal34Met mutations have a type I and a type IIb heterozygous PC deficiency.

To analyze the PC-binding domain of EPCR, we sequenced exons 2 and 3 of PROCR, which encode for the PC-binding domain of EPCR. PCR amplification and sequencing of exons 2 and 3 were successful in all controls and in 630 of 653 and 649 of 653 patients, respectively. No SNVs were detected in exon 2 of PROCR in either the patients or the controls. As shown in Table 3, the activation rate of WT PC is =4-fold higher on HEK293 cells expressing EPCR and TM (gray bars) compared with that observed on HEK293 cells (white bars). Preincubation of the cells with the anti-EPCR antibody that blocks PC–EPCR interactions (JRK 1535; 500 nmol/L) inhibits the activation rate of WT PC on HEK293/EPCR/TM cells. The activation rate of PCArg9Cys is similar to that of WT PC, whereas PCArg-1Cys and PCVal34Met have impaired capacity to generate APC. Thus, patients carrying the PCArg-1Cys and PCVal34Met mutations have a type I and a type IIb heterozygous PC deficiency.

To analyze the PC-binding domain of EPCR, we sequenced exons 2 and 3 of PROC, which encode for the PC-binding domain of EPCR. PCR amplification and sequencing of exons 2 and 3 were successful in all controls and in 630 of 653 and 649 of 653 patients, respectively. No SNVs were detected in exon 2 of PROC in either the patients or the controls. As shown in Table 3, we found 3 SNVs in exon 3 that occurred in the patients but not in the controls. The first SNV was a C to T transition at nt 6367, which results in a Arg96Cys substitution. The second SNV was a G to C transition at 6589, which results in a Val170Leu substitution. The third SNV was a silent mutation (C6519T). Within intron 2, we found 3 SNVs (G5212A, G5195A, and C5334A) in the patients but not in the controls. Within intron 3, we found a SNV (G deletion at 6738) that is present in both the patients and the controls, and a SNV (A6668T) that is present at a higher frequency in the patients than in the controls.

To determine whether the EPCR SNVs result in abnormal proteins with reduced binding affinity for PC, the cDNAs of

<table>
<thead>
<tr>
<th>SNV or Insertion</th>
<th>Consequence</th>
<th>HGVS Designation</th>
<th>Consequence</th>
<th>Mutation Database</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROC Exon 3 C 2896 T Arg-1Cys</td>
<td>NM_000312.3:c.124C&gt;T</td>
<td>p.Arg42Cys</td>
<td>HGMD CM930604, ProCMD variant # 13</td>
<td></td>
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<tr>
<td>PROC Exon 3 C 2923 T Arg9Cys</td>
<td>NM_000312.3:c.151C&gt;T</td>
<td>p.Arg51Cys</td>
<td>HGMD CM950976, ProCMD variant # 18</td>
<td></td>
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<tr>
<td>PROC Exon 3 G 2998 A Val34Met</td>
<td>NM_000312.3:c.226G&gt;A</td>
<td>p.Val76Met</td>
<td>HGMD CM920593, ProCMD variant # 29</td>
<td></td>
</tr>
<tr>
<td>PROC Intron 2 C 2633 G</td>
<td>NM_000312.3:c.70+1061C&gt;G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROC Intron 2 C 2730 T</td>
<td>NM_000312.3:c.70+1158C&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROC Intron 3 G 3310 A</td>
<td>NM_000312.3:c.237+301G&gt;A</td>
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</table>

**Table 1. Human Genome Variation Society Nomenclature of Single Nucleotide Variants in PROC and PROCR**

**Table 2. Summary of SNVs Identified in Exon 3, Intron 2, and Intron 3 of PROC**

<table>
<thead>
<tr>
<th>SNV</th>
<th>Location</th>
<th>Consequence</th>
<th>No. of Patients (n=653)</th>
<th>No. of Controls (n=627)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 2896 T Exon 3</td>
<td>Arg to Cys at position −1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C 2923 T Exon 3</td>
<td>Arg9Cys</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G 2998 A Exon 3</td>
<td>Val34Met</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C 2633 G Intron 2</td>
<td></td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C 2730 T Intron 2</td>
<td></td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>G3310A Intron 3 G/G</td>
<td>65.9%</td>
<td>G/G 395 (63%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3310A Intron 3 G/A</td>
<td>30.0%</td>
<td>G/A 210 (33.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3310A Intron 3 A/A</td>
<td>4.6%</td>
<td>A/A 22 (3.5%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNV indicates single nucleotide variant.
WT EPCR and the 2 EPCR variants were stably transfected into HEK293 cells, and the ability of the EPCR variants to bind to fluorescently labeled APC was measured by flow cytometry. All 3 stable cell lines expressed similar levels of EPCR on the cell surface as confirmed by flow cytometry using an anti-EPCR antibody (JRK 1535; Figure 4). WT EPCR bound to fluorescently labeled APC with a $K_d$ value of 77±21 nmol/L, consistent with previous reports. In contrast, EPCR variants, Arg96Cys and Val170Leu, bound to fluorescently labeled APC with reduced affinities ($K_d$ values ≥2 µmol/L, which is higher than the circulating concentration of PC $\approx 60$ nmol/L). The binding curves obtained from the flow cytometry studies are shown in Figure 5.

We also determined the prevalence of the previously reported PROCR A3 haplotype. This haplotype results in substitution of Ser219 with Gly in the transmembrane domain of EPCR, which leads to increased endothelial shedding of EPCR and reduced EPCR function. The PROCR A3 haplotype occurred at a significantly higher frequency in the patient group compared with the control group ($P=0.001$; Table 4). It should be noted that none of the carriers of the PROCR A3 haplotype were carriers of the PC or EPCR coding region SNVs described above.

Finally, we identified a 23-bp insertion in exon 3 of PROCR in 1 control individual, but not in any of the patients with VTE (insertion described in Table 1). Previous studies have shown that this insertion codes for 5 amino acids (YPQFL), which is followed by a stop codon, resulting in an abnormal EPCR protein that lacks part of the extracellular domain, the transmembrane domain, and the cytoplasmic tail.

**Discussion**

In this study, we explored the possibility that genetic mutations that impair PC–EPCR interactions may be associated with an increased risk of VTE. With respect to PROC, we identified 3 SNVs, each in individual patients, that result in mutations in the Gla domain of PC (PCArg-1Cys, PCArg9Cys, and PCVal34Met). The most common thrombophilic defects in the ELATE patients were Factor V Leiden, the prothrombin G20210A gene mutation, and antithrombin deficiency, with prevalences of 26.5%, 9.3%, and 3.6%, respectively. It should be noted that none of these 3 patients were carriers of the

**Figure 1.** Western blot analysis of protein C (PC) variants expressed in human embryonic kidney (HEK) 293 cells. HEK293 cells were stably transfected with cDNAs encoding either wild-type PC (WT PC) or PC variants (PCArg-1Cys, PCArg9Cys, and PCVal34Met). All cell lines contained similar levels of PC mRNA as assessed by the measurement of real-time polymerase chain reaction. The cell lysates and conditioned medium were separated by SDS-PAGE (4%–15% gradient gel) under reducing conditions and subjected to Western blot analysis with HPC4, a monoclonal antibody against human PC. The nitrocellulose membrane was stained with Ponceau S dye to confirm that similar amounts of protein were loaded in each lane (sample section of the nitrocellulose membrane is shown).

**Figure 2.** Protein C (PC) antigen levels in healthy volunteers and in the Extended Low-intensity Anticoagulation for Thromboembolism (ELATE) Study patients (with or without PROC Gla domain mutations). PC antigen levels were measured in plasma samples from 6 healthy volunteers and in 21 warfarin-treated ELATE Study patients (8 patients had mutations in the Gla domain of PC and 18 did not). $^*P<0.05$; and $^{**}P<0.001$.

**Figure 3.** Analysis of wild-type human protein C (WT PC) and PC variants in PC activation assays. PC activation assays were performed as described in Materials and Methods in the online-only Data Supplement. Gray bars, PC activation rates in the presence of human embryonic kidney (HEK) 293 cells expressing endothelial PC receptor (EPCR) and thrombin–thrombomodulin. White bars, PC activation rates in the presence of HEK293 cells. JRK 1535 (an antibody that inhibits the binding of PC to EPCR) was used at a concentration of 500 nmol/L. The bars represent the mean, and the lines above the bars reflect the SE of ≥3 determinations. $^*P<0.05$ compared with WT PC.
Factor V Leiden gene, the prothrombin gene mutations, or had antithrombin deficiency.

All 3 PROC SNVs have been reported previously (HGMD CM930604; HGMD CM950976; and HGMD CM920593). In this study, we showed that the 3 PC variants displayed reduced protein synthesis when expressed in HEK293 cells (Figure 1), and thus are classified as type I deficiencies. Consistent with this observation, the PC antigen levels in the ELATE Study patients harboring the Val34Met, Arg9Cys, and Arg-1Cys mutations are lower than those in healthy volunteers and in the ELATE Study patients without PC mutations (Figure 2). It is possible that warfarin may have reduced PC antigen levels in the 3 patients with, and the 18 patients without, the PROC mutations; however, the comparison of PC antigen levels between these 2 groups, and the finding of lower PC antigen levels in the 3 patients with the PROC mutations, should still be valid. Interestingly, the variants are secreted as a lower molecular weight form of \( \approx 40 \text{ kDa} \) (Figure 1). PC possesses 4 N-linked glycosylation sites: 1 is located in the first EGF domain (Asn 97), and 3 are located in the serine protease domain (Asn248, Asn313, and Asn 329).\(^{18}\) α-PC, which is glycosylated at all 4 sites, accounts for \( \approx 70\% \) of plasma PC. In contrast, β-PC, which lacks glycosylation at Asn 329 and thus has a lower molecular weight, accounts for \( \approx 30\% \) of plasma PC. Previous studies suggest that the degree of glycosylation at Asn 329 is influenced by the correct post-translational processing of the Gla domain.\(^{21}\) Thus, the presence of mutations within the Gla domain of the PC variants likely favors the formation of the β-form of PC. In patients, the presence of only the β-form of PC in plasma results in decreased anticoagulant activity.\(^{22}\) Specifically, the naturally occurring PCN329T mutation results in an abnormal APC with impaired ability to inactivate Factor Va.

<table>
<thead>
<tr>
<th>SNV</th>
<th>Location</th>
<th>Consequence</th>
<th>No. of Patients</th>
<th>No. of Controls</th>
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<tr>
<td>C 6367 T</td>
<td>Exon 3</td>
<td>Arg96Cys</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G 6589 C</td>
<td>Exon 3</td>
<td>Val170Leu</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C 6519 T</td>
<td>Exon 3</td>
<td>Silent (Phe to Phe)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>G 5212 A</td>
<td>Intron 2</td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>G 5195 A</td>
<td>Intron 2</td>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C 5334 A</td>
<td>Intron 2</td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>A6668T</td>
<td>Intron 3</td>
<td></td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>G deletion at 6738</td>
<td>Intron 3</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

SNV indicates single nucleotide variant.
The PCArg-1Cys and PCVal96Met variants, both of which carry a free cysteine residue, have been shown to form a complex with α1-microglobulin.21 Although we did not observe complexes between these PC variants and α1-microglobulin based on our Western blot analysis (presumably because HEK293 cells do not synthesize α1-microglobulin), it is possible that these complexes exist in the patient plasma.

We also observed that the PCArg-1Cys and PCVal96Met variants (secreted and purified from HEK293 cells) do not synthesize α1-microglobulin. Although we did not observe complexes between these PC variants and α1-microglobulin, the current study demonstrated that introduction of either EPCR Arg96Cys or the Val170Leu mutation results in abnormal EPCR protein with impaired ability to bind PC. Residues Arg96 and Val170 are located near residues that form hydrogen bonds between EPCR and Gla residues of PC.12,13

Although these EPCR variants do not bind to PC, they retain the ability to bind to JRK 1535 (a monoclonal antibody to EPCR), suggesting that the tertiary structure is maintained. In baboons, pretreatment with a monoclonal antibody to EPCR (which blocks PC binding) reduced the amount of thrombin-induced APC generation by 88%.2 EPCR gene disruption in mice results in early embryonic lethality because of placental thrombosis.26

Other polymorphisms in PROCR have been described. The first reported abnormality was a 23-bp insertion in exon 3 that leads to the production of a truncated EPCR, which does not bind to PC.20 In the present study, we found this insertion in one of our control subjects. However, the clinical impact of this mutation is difficult to assess because of its low allelic frequency.20,27–30 More recent studies have shown that soluble EPCR levels occur with a bimodal distribution in the normal population; ≈80% of subjects have low levels of soluble EPCR (<180 ng/mL), whereas ≈20% have high levels (between 200 and 700 ng/mL).31,32 Analysis of PROCR revealed that the A3 haplotype is over-represented in patients with VTE compared with the controls.15 The A3 haplotype results in a Ser to Gly substitution in the transmembrane domain of EPCR that promotes cellular shedding of EPCR in endothelial cells.33 In the current study, we confirmed that the EPCR A3 haplotype occurs at a significantly higher frequency in the patient group compared with the control group (P<0.001).

There are limitations to our study. First, the probability of finding 5 variants in the cases, but not in the controls, is P=0.062 (2-tailed Fischer exact test). Thus, the results of this study should be validated in larger studies of patients with unprovoked VTE. Second, the control population was younger in age than the ELATE patient. However, the cases and controls do have similar ethnicity and geographical background (predominantly whites), which may prevent false-positives because of population stratification.

To date, no common single nucleotide polymorphisms in the PROC and PROCR genes have been identified in genome-wide association studies of VTE.34–36 The results of a discovery genome-wide association studies for VTE in 1618 VTE cases of European origin confirmed well-known association of the FV Leiden variant and the ABO O blood group with VTE.34–36 Additional genome-wide significant signals at the F11 region were associated with an increased risk of thrombosis.36 Because genome-wide association studies cannot detect rare variants, the missing heritability for VTE risk may be because of clusters of rare variants. Our study suggests that rare variants in PROC and PROCR are more likely to be found in unprovoked VTE cases than in controls. Our data support the common disease, rare variant hypothesis that multiple rare variants, with functional effects and relatively high penetrance, are the major contributors to genetic susceptibility to a complex disease, such as VTE.37,38 Thus, high-throughput sequencing studies of candidate genes may emerge as a more accurate and powerful tool to elucidate the relationship between genetics and VTE.

**Table 4. Frequency of the A3 Haplotype in PROCR**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non-A3 Haplotypes</th>
<th>A3 Haplotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total A3 Haplotype</td>
<td></td>
</tr>
<tr>
<td>ELATE patients (n=657)</td>
<td>497 (77.1%)</td>
<td>137 (21.2%)</td>
</tr>
<tr>
<td>Controls (n=627)</td>
<td>501 (86.5%)</td>
<td>72 (12.4%)</td>
</tr>
</tbody>
</table>

*P<0.001 for Extended Low-intensity Anticoagulation for Thrombo-embolism (ELATE) patients compared with all controls.
In summary, this is the first targeted DNA sequencing analysis of \textit{PROC} and \textit{PROCR} in a large group of patients with unprovoked VTE. We identified 5 SNVs in the coding regions of \textit{PROC} and \textit{PROCR} that are present in the patients, but not in the controls. Expression of the mutant proteins revealed impaired synthesis and/or reduced capacity for PC–EPCR interactions. We also validated previous findings that the EPCR A3 shedding haplotype is over-represented in patients with VTE. Our data suggest that genetic mutations that impair PC–EPCR binding interactions may increase the risk of VTE.

\section*{Acknowledgments}
We thank Jenny Nguyen for technical assistance with the DNA sequence analysis in this study. We also thank the ELATE study investigators and the subjects who were enrolled in the ELATE study for making this study possible.

\section*{Sources of Funding}
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\section*{Disclosures}
None.

\section*{References}


The endothelial protein C receptor (EPCR) is an important cofactor for the conversion of protein C (PC) to the anticoagulant enzyme activated PC on the endothelial cell surface. We hypothesized that mutations that impair PC–EPCR interactions may be associated with an increased risk of venous thromboembolism. We sequenced portions of the PROC and PROCR genes in 653 patients with unprovoked venous thromboembolism and in 627 healthy controls. Five single nucleotide variants were identified that result in abnormal PC (Arg9Cys, Val34Met, and Arg1Cys) or abnormal EPCR proteins (Arg96Cys and Val170Leu) in the patients. None of these single nucleotide variants were found in the controls. Expression of these mutants in human embryonic kidney 293 cells revealed impaired synthesis and reduced capacity for PC–EPCR interactions. In addition, the previously reported EPCR A3 haplotype, which promotes cellular shedding of EPCR, is over-represented in the patient group. This is the first targeted DNA sequencing analysis of the PROC and PROCR genes in a large group of patients with unprovoked venous thromboembolism. Our data suggest that mutations that impair PC–EPCR interactions may be associated with an increased risk of venous thromboembolism.
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MATERIALS AND METHODS

Patients and controls- A total of 738 predominantly white patients from 16 clinical centers were enrolled in the ELATE trial (Extended Low-intensity Anticoagulation for Thrombo-Embolism), which compared low-intensity and conventional-intensity anticoagulation with warfarin for the prevention of recurrent VTE\(^1\). These were patients with one or more episodes of unprovoked VTE who had completed at least 3 months of conventional-intensity oral anticoagulant therapy. Unprovoked VTE was defined as objectively confirmed, symptomatic, proximal deep venous thrombosis or pulmonary embolism that occurred in the absence of a major risk factor for thrombosis (e.g. fracture or plaster casting of leg, or hospitalization with confinement to bed for three consecutive days, in the 3 months before thrombosis; active cancer within the previous two years). Of the 738 patients who were enrolled in the ELATE trial, 653 patients (88%) had consented to provide blood samples and were included in the current study. We have previously reported the prevalence of common thrombophilias in these patients, and the association between these abnormalities and recurrent VTE while on anticoagulant therapy\(^2\). The ELATE trial and the current study were approved by the institutional review boards of all participating centers, and written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

A total of 627 control subjects with no known history of VTE were obtained from three groups. The first group consisted of 273 young, healthy white individuals (mainly college students) who participated in the Genes and Blood Clotting Study (GABC)\(^3\). This group had a median age of 21 ± 3.5 years and 65% were female. 76% self-identified as white while 8.8% were of Asian ancestry, 5.5% were African American and 5.2% were Spanish, Hispanic or Latino. The second group consisted of 288 white individuals referred for hereditary
hemochromatosis (HFE gene) testing. The mean age in the second group was 59 ± 12 years and 44% were female. Of these, HFE testing was positive in 17% (to our knowledge, hemochromatosis is not known to be associated with VTE). The third group consisted of 66 white individuals from Hamilton, Ontario, Canada (mainly graduate students and laboratory technicians) with a mean age of 45 ± 11 yrs and 39% were female.

**DNA sequence analyses of PROC and PROCR**- Blood was collected from patients and controls by venipuncture into vacutainer tubes containing 0.105 M trisodium citrate pH 5.4 (1 vol/9 vol blood). Genomic DNA was extracted from white blood cells using the QIAamp DNA Blood Minikit (Qiagen Inc., Valencia, CA). The primers used to PCR amplify the 650 bp fragment encompassing the coding region for the PC Gla domain are as follows: 5’ TAG AAA GGT AAA GAC ACT GGC 3’ and 5’ GAA GCA GGG TGG TCT TTA GGT 3’. To study the PC binding domain of EPCR, we PCR amplified portions of exons 2 and 3 of PROCR. The primers for EPCR exon 2 are: 5’ CAG CGT CAA ACG GGA GAA GC 3’ and 5’ AGA GGT TAT GCC AGC AGG ACG 3’. The primers for EPCR exon 3 are: 5’ CTC TCC TCA CAG CAC TGA CTC 3’ and 5’ CCT TGC CAG CCT CCA TCA ATC 3’. The exon 3 primers also allowed us to amplify a portion of exon 4 which contains the PROCR A3 haplotype polymorphism site. The PCR amplification products were analyzed on an ABI Prism 3700 sequencer (MOBIX Sequencing Core Facilities, McMaster University).

**Mutagenesis of PC and EPCR genes**- Human EPCR cDNA4 was cloned as a XhoI/NotI fragment into the multiple cloning site of the eukaryotic expression vector pcDNA3.1(-) (Invitrogen, San Diego, CA). Human protein C cDNA was cloned as a HindIII/XbaI fragment into the multiple cloning site of pcDNA3.1(+) (kindly provided by Dr. Alireza Rezaie, Dept. of Biochemistry, St. Louis University). *In vitro* mutagenesis to generate and select point mutations
was performed using the QuickChange site-directed mutagenesis system as described by the supplier (Stratagene, La Jolla, CA). Double-stranded DNA sequencing was used to verify the authenticity of the mutations.

**Stable expression of variant forms of PC or EPCR in HEK293 cells** - PC or EPCR variants were stably transfected and expressed in HEK293 cells as previously described. Transfection of HEK293 cells with the cDNA of PC variants (PCArg-1Cys, PCArg9Cys, PCVal34Met) was performed in 10-cm dishes using the Effectene transfection reagent as described by the supplier (Qiagen). The cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM) (Life Technologies Inc., Burlington, ON) supplemented with 10% fetal bovine serum (FBS) (Life Technologies Inc.) and 5 µg/ml vitamin K1 (Sigma-Aldrich, St. Louis, MO). 48 h post-transfection, the medium was changed to DMEM containing 10% FBS, 5 µg/ml vitamin K1, and 400 µg/ml G418 (Life Technologies Inc.). After 2 to 3 weeks of drug selection in which the medium was changed every day, drug-resistant colonies were isolated.

**Purification of PC variants stably expressed in HEK293 cells** - Stable cell lines expressing the PC variants were grown in 24 x 24 cm dishes containing DMEM, 10% FBS, 1 mg/ml G418, penicillin (100 U/ml)-streptomycin (100 µg/ml), and 5 µg/ml vitamin K until 80% confluence. The cells were washed 3 times with PBS and grown for 48 hr in serum-free DMEM containing G418, penn-strep, and vitamin K. The PC variants were purified by immunoaffinity chromatography using the Ca$^{2+}$-dependent anti-human PC monoclonal antibody (HPC4) linked to Affigel-10 (Biorad) as previously described.

**Real-time PCR quantitation of PROC and β-actin mRNA levels** - Total RNA was extracted from HEK293 stable cell lines expressing WT PROC or PROC variants using the RNeasy Mini Mammalian Total RNA kit (Qiagen, Mississauga, ON) as per manufacturer’s instructions. The
synthesis of cDNA was performed using the SuperScript III First Strand Synthesis System for RT-PCR with random hexamer primers (Invitrogen) according to the manufacturer’s protocol. Real-time PCR analysis of PROC and β-actin mRNA was performed using SYBR GreenER qPCR Supermix (Applied Biosystems) as per manufacturer’s instructions using the Step One Plus machine (Applied Biosystems). Specific primers were used to amplify PROC and β-actin mRNA (McMaster University MOBIX Laboratory). The primer sequences are as follows:

PROC Forward: 5’-TAGAAAGGTAAGACACTGGC-3’
PROC Reverse: 5’-GAAGCAGGGGTGGCTTTAAGGT-3’
β-actin Forward: 5’-ACC GAG CGC GGC TAC AG-3’
β-actin Reverse: 5’-CTT AAT GTG ACG CAC GAT TTC C -3’

**PC ELISA**- Venous blood (4.5 mL) was drawn via venipuncture from patients or control subjects into vacutainer tubes containing 0.5 mL 0.105 M buffered trisodium citrate (pH 5.4). The blood was spun at 1500x g for 10 minutes at 20°C, and the plasma was stored as aliquots at -80°C. PC antigen in the plasma samples were quantified by a sandwich-type enzyme immunoassay (Affinity Biologicals, Ancaster, ON, Canada). The capturing antibody is a Ca$^{2+}$-independent monoclonal antibody (Product # PC-EIA).

**PC activation assays**- Protein C activation assays were performed using HEK293 cells stably expressing EPCR and TM as previously described$^7$. Briefly, the cells were washed 3 times with phosphate-buffered saline. The cells were preincubated for 5 min at room temperature with 0.5 ml of HBSS containing 25 mM Hepes, pH 7.5, 0.1% bovine serum albumin, 3 mM CaCl$_2$, and 0.6 mM MgCl$_2$ before the addition of 100 nM WT PC or the PC variants (PCArg-1Cys, PCArg9Cys, PCVal34Met). PC activation was initiated by the addition of 10 nM thrombin. In
some cases, 500 nM anti-EPCR mAb JRK 1535 was added before the addition of WT PC and 
preincubated with the cells for 10 min at room temperature. After 30 min at 37 °C, 100 ml of the 
reactions were stopped by the addition of 20 ml of 1.66 mg/ml antithrombin containing 20 mM 
EDTA. 50 µL of the supernatant was transferred into a 96-well microplate, and amidolytic 
activities of APC were determined toward 0.2 mM Spectrozyme PCa substrate in 20 mm Tris- 
HCl, pH 7.5, 150 mM NaCl. The rates of substrate cleavage were measured in a $V_{\max}$ 
microplate reader (Molecular Devices) and were linear over time. All determinations were 
performed in triplicate.

**Western Blot Analysis of PC variants expressed in HEK293 cells**—HEK293 cells stably 
transfected with the cDNAs encoding the PC variants were grown in 10 cm dishes and serum 
starved for 48 hours. The cells were lysed in 5 ml of phosphate-buffered saline containing 1% 
Triton X-100 at room temperature for 10 min. The levels of PC variants in the cell lysates and 
conditioned medium were analyzed by electrophoresis and immunoblotting. Electrophoresis was 
performed under reducing conditions according to the method of Laemmli using 4-15% SDS-
polyacrylamide gels. Immunoblotting was performed using HPC4, a Ca$^{2+}$-dependent anti-human 
PC monoclonal antibody (kindly provided by Dr. Charles Esmon, Oklahoma Medical Research 
Foundation).

**Flow Cytometric Analysis of Fl-APC Binding to EPCR Mutants**—APC was labeled at the 
active site with fluorescein (Fl-APC) as previously described. HEK293 cells stably transfected 
with EPCR variants were grown to confluence in T-75 flasks, detached with gentle pipetting, and 
suspended in 3 ml of complete HBSS buffer (HBSS containing 25nM HEPES pH 7.5, 3 mM 
CaCl$_2$, 0.6 mM MgCl$_2$, 1% bovine serum albumin). The cells were diluted 1:20 in complete 
HBSS buffer and incubated with increasing concentrations of Fl-APC at 4 °C for 15 min in the
dark. Bound Fl-APC was detected on the fluorescence-1 channel on a FACSCalibur flow cytometer (Becton Dickinson). The fluorescence intensity of each sample was analyzed twice. Values for \( K_d \) were determined by fitting binding isotherms with a hyperbolic equation using the TableCurve program (Jandel Scientific, San Rafael, CA).
REFERENCES FOR MATERIALS AND METHODS


