Protein C, Antithrombin, and Venous Thromboembolism Incidence
A Prospective Population-Based Study

Aaron R. Folsom, Nena Aleksic, Lu Wang, Mary Cushman, Kenneth K. Wu, Richard H. White

Abstract—Although deficiencies of protein C and antithrombin, 2 natural plasma anticoagulants, are known risk factors for venous thrombosis, population-based prospective incidence data on these associations are lacking. Venous thromboembolic events have been identified in adults in 2 longitudinal cohort studies, the Atherosclerosis Risk in Communities (ARIC) Study and the Cardiovascular Health Study (CHS). Incidence was examined in relation to prediagnostic plasma levels of protein C (ARIC Study only) and antithrombin. Over a mean of 8.1 years of follow-up, there were 130 incident venous thromboembolic events that were not due to cancer in the ARIC Study. The age-adjusted incidence was elevated 3.36-fold (95% CI 1.24 to 9.11) in the 1.1% of subjects with protein C values <2.0 mg/L compared with subjects with higher values. In contrast, in the ARIC Study and the CHS, there was no association between low plasma antithrombin and venous thromboembolism. In conclusion, in this population-based study, a low protein C, but not antithrombin, level has been determined to be associated with an increased incidence of venous thromboembolism. Attributable risk estimates suggest that low protein C levels account for ≈2.5% of venous thromboembolic events in the ARIC population. (Arterioscler Thromb Vasc Biol. 2002;22:1018-1022.)

Key Words: antithrombin ■ protein C ■ prospective study ■ venous thrombosis ■ pulmonary embolus

Deficiencies of protein C and antithrombin, 2 natural plasma anticoagulants, are known contributors to familial venous thrombosis. Type I deficiency is defined by the reduction of the functional activity and plasma antigen levels of the anticoagulant; type II deficiency is defined by reduced activity but normal antigen levels. Deficient protein C or antithrombin is typically present in 4% to 9% of the patients with familial thrombophilia.1 In unselected patients with venous thrombosis, deficiencies of protein C and antithrombin are less common (≈3% and 1%, respectively).2–4 In contrast, the prevalence of protein C deficiency in the general population has been estimated to be between 0.2% and 0.4%,5,6 and the prevalence of type I antithrombin deficiency has been estimated to be 0.02%.7 A number of mutations of the protein C and antithrombin genes contribute to deficiencies of these anticoagulant proteins.8 In middle-aged carriers of such mutations, the incidence of venous thrombosis is ≈1% per year and is somewhat higher for antithrombin than for protein C deficiency.8–11 Lifestyle and other physiological factors contribute modestly to variations in protein C and antithrombin levels in the population.12,13

Rosendaal14 has estimated the population attributable risk (ie, the proportion of venous thrombosis caused by a risk factor) to be 2% for protein C deficiency and <1% for antithrombin deficiency and much less for activated protein C resistance or elevated factor VIII. To our knowledge, there has been no longitudinal study of the general population relating levels of protein C or antithrombin to incidence of venous thromboembolism (VTE). We undertook the present study to test the hypothesis that low levels of these natural anticoagulants are associated with increased VTE incidence in the general population.

Methods

Study Population and Baseline Assessments

The Longitudinal Investigation of Thromboembolism Etiology (LITE) Study is a prospective study of VTE occurrence in 2 pooled, multicenter, longitudinal, population-based cohort studies: the Atherosclerosis Risk in Communities (ARIC) Study and the Cardiovascular Health Study (CHS). The LITE Study design, methods, and VTE incidence rates have been described in detail elsewhere.15,17 In brief, 15 792 ARIC participants aged 45 to 64 years at baseline in 1987 to 1989 and 5201 CHS participants aged ≥65 years at baseline in 1989 to 1990 underwent assessments of cardiovascular risk factors. An additional 687 African Americans were recruited to CHS in 1992 to 1993. Baseline cardiovascular risk factors included in the present study were measured comparably in the ARIC Study and the CHS, as described elsewhere.18

After informed consent was obtained, blood was drawn from fasting participants in the morning in both studies. The blood

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From the Division of Epidemiology (A.R.F., L.W.), School of Public Health, University of Minnesota, Minneapolis; the Division of Hematology (N.A., K.K.W.), University of Texas Medical School, Houston; the Department of Medicine (M.C.), University of Vermont, Burlington; and the Division of General Medicine (R.H.W.), University of California-Davis, Sacramento.
Correspondence to Aaron R. Folsom, MD, Division of Epidemiology, School of Public Health, University of Minnesota, Suite 300, 1300 South Second St, Minneapolis, MN 55454-1015. E-mail folsom@epi.umn.edu
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samples were stored briefly on ice (ARIC Study) and at room temperature (CHS), then centrifuged for 3000g for 10 minutes, and stored in −70°C freezers. Up to 3 follow-up examinations were performed every 3 years in the ARIC Study, and up to 9 follow-up examinations were performed annually in the CHS. Blood samples from the subsequent examinations were stored at −70°C.

**VTE Case Identification**

Potential cases of VTE were identified from baseline through September 1998. Hospital records were obtained, and VTE events were validated by 2 physicians as “definite deep vein thrombosis (DVT)” (nearly always having a positive duplex ultrasound or a positive venogram), “probable DVT” (having a positive Doppler ultrasound or a positive impedance plethysmography), and “definite PE” (having ventilation-perfusion scans with multiple segmental or subsegmental mismatched defects, a positive pulmonary angiogram, or CT or autopsy documentation of pulmonary embolism [PE]). Definite and probable DVT and definite PE were pooled as VTE for this analysis.

Cases were also classified as incident (no self-reported VTE history before baseline) or recurrent (self-reported VTE history before baseline) and as idiopathic (no obvious cause) or secondary (associated with cancer, major trauma, surgery, or marked immobility). In the ARIC Study, 185 individuals developed VTE; 150 developed VTE in the CHS. Of the 335 events, 237 had venous thrombosis only, 52 had PE only, and 46 had both.

**Study Designs**

Two study designs were used to accomplish our aims. In the ARIC Study, but not in the CHS, plasma protein C and antithrombin were measured for all participants, thus allowing a traditional cohort analysis. Because the CHS had not measured these factors, we decided to measure antithrombin, the rarer of the 2 deficiencies, on all cases and controls from the LITE Study (ie, remeasured in the ARIC Study and first-measured in the CHS). This subcohort is referred to as the “LITE case and control subset.” We also measured protein C in the ARIC and CHS cohorts at a ratio of 2:1 per VTE case and were frequency-matched to the cases by age (5-year groupings), sex, race (black or white), follow-up time (case’s event date within 2 years of control’s assigned date), and study (ARIC Study or CHS).

Stored samples of DNA and plasma for cases and controls were retrieved from storage freezers. If baseline plasma samples were limited, previously thawed, or exhausted for a participant, a sample was retrieved from the plasma repository for the next visit (~3 years after baseline); if neither sample was available, it was considered missing. The percentages of ARIC subjects having plasma from baseline, the year 3 visit, or missing were 65%, 25%, and 10%, respectively. The respective percentages for CHS plasma were 80%, 14%, and 6%. The percentage of missing samples did not significantly differ between cases and controls. For only 12 cases in the LITE Study, we used blood drawn after the VTE. For factor V Leiden, 8% of the ARIC participants and 10% of the CHS participants had missing samples or refused consent for use of their DNA.

**Laboratory Methods**

Protein C antigen was measured at the ARIC baseline by using a commercial ELISA kit (Asserachrom Protein C, Diagnostica Stago). Low protein C values were repeated and confirmed. The coefficient of variation for protein C was 12%; the reliability coefficient (between-subject variance divided by total variance) obtained from repeated testing of individuals over several weeks was 0.56.

Antithrombin was assayed at the ARIC baseline with an amidolytic assay using a synthetic chromogenic substrate for thrombin (CBS 34.47, Diagnostica Stago) and bovine thrombin (Kabi). The test may be influenced by heparin cofactor II. The antithrombin values (percentages) were obtained by relating the optical density of the unknown samples to a calibration curve that was constructed with each run by use of a hromatized plasma pool (University Coagulation Reference Plasma ThromboScreen, Pacific Hemostasis) as the standard.

Low antithrombin values (values <50%) were repeated and confirmed. The coefficient of variation for antithrombin was 12%; the reliability coefficient assessed by repeat visits over several weeks was 0.42.

Antithrombin was measured in the LITE Study (remeasured in the ARIC Study and first-measured in the CHS) by use of an automated chromogenic assay (IL Test Antithrombin) based on bovine factor Xa; an ACL Futura Coagulation System (Instrumentation Laboratory) was used. The method is specific and is not influenced by heparin cofactor II. Within-run coefficients of variation were 3% for normal values and 4% for low values of antithrombin. Low values of antithrombin were repeated and confirmed. The reliability coefficient based on 15 split specimens, which were stored for a long time and then analyzed, was r = 0.38. Because the ARIC Study had stored samples briefly on ice and the CHS had stored samples at room temperature, we conducted a pilot study (n = 10) processing split normal plasma samples both ways. The mean ± SD antithrombin values were 106 ± 9 for room temperature and 110 ± 12 for cold processing (r = 0.72).

We detected the presence or absence of the factor V Leiden (1691G→A, R506Q) mutation and the prothrombin 20210G→A polymorphism on LITE cases and controls by using standard methods. Factor VIII coagulant activity (VIIIc) was measured by a Coag-a-MateX2 (Organon-Teknika) with the use of factor immuno-deficient plasma (Organon-Teknika).

**Statistical Analyses**

A traditional longitudinal analysis was used to analyze protein C and antithrombin measured at baseline in the ARIC Study. Because the focus was on incident VTE–unrelated cancer, we first excluded 276 participants who reported at baseline a history of VTE, 874 who reported a history of cancer, 87 who reported baseline anticoagulant use, and 273 who had missing blood measurements (a total of 1434 were excluded; some had >1 exclusion). This left 14 358 subjects at risk to be followed longitudinally. Rates of VTE not due to cancer were calculated for 2 definitions of low protein C and antithrombin, namely, <1 percentile (2.0 mg/L for protein C and <65% for antithrombin) and <5 percentile (2.3 mg/L for protein C and <80% for antithrombin). Relative risks (RRs [hazard ratios]) and 95% CIs were calculated by using Cox proportional hazards models. The dose-response relation for protein C was examined graphically by using a restricted cubic spline model. The hazard ratio was plotted relative to a value of 3.7 mg/L, which was approximately the 80th percentile.

For antithrombin in the LITE nested case-control sample, we also restricted analyses to incident events and excluded 51 participants with a history of VTE, 130 with a history of cancer, and 19 with baseline anticoagulant use. There were 219 cases (the ARIC Study plus the CHS) and 529 controls remaining for analyses. We used unconditional logistic regression to calculate odds ratios and 95% CIs of VTE in relation to antithrombin <1 percentile (55%) and <5 percentile (65%).

A number of lifestyle, physiological, and hemostatic factors have already been evaluated for their association with VTE in the LITE cohort. Adjustment was made in the ARIC and LITE analyses for factors previously associated with VTE, including age (continuous) in all models and race, sex, body mass index (continuous), and factor VIIIc (continuous) in multivariate models.

**Results**

The ARIC cohort at baseline was aged 45 to 64 years (mean 54 years); 72% were white, and 28% were black. The LITE cases of VTE and controls at baseline were aged 45 to 94 years (mean 64 years); 74% were white, and 26% were black. Approximately 14% of cases and 4% of controls carried factor V Leiden, and 4% of cases and 2% of controls carried the prothrombin 20210A polymorphism.

In the ARIC baseline cohort in 1987 to 1989, the mean ± SD for protein C was 3.17 ± 0.62 mg/L; for antithrombin.
bin, it was $111 \pm 22\%$. By use of a different assay and stored samples, the baseline mean antithrombin was $90 \pm 16\%$ in LITE VTE cases and controls. By the different assays, the 2 antithrombin values for the ARIC participants were correlated ($r = 0.36$).

Over a mean of 8.1 years of follow-up, there were 130 incident VTE events unrelated to cancer in the ARIC Study (incidence rate 1.10 per 1000 person-years). The prevalence of protein C values $<2.0$ mg/L was 3.2% ($n=4$) among the VTE cases versus 1.1% ($n=156$) in all of the ARIC Study. Compared with higher values, the age-adjusted incidence of VTE was elevated 3.36-fold in subjects with protein C values $<2.0$ mg/L (Table). With adjustment for age, race, sex, body mass index, and factor VIIIc, this RR was unchanged (3.38, 95% CI 1.24 to 9.20). VTE incidence was elevated only 1.19-fold for protein C values $<2.3$ mg/L (Table).

By use of a cubic spline method (Figure), it appeared that the RR of VTE began rising at protein C values $<2.5$ mg/L and that it might rise to an RR of 4 at protein C levels $\leq 1.5$ mg/L. (A total of 33 ARIC subjects had protein C values $\leq 1.5$ mg/L.) VTE risk was fairly constant at protein C values $>2.5$ mg/L.

Low antithrombin, in contrast, did not seem to be associated with the incidence of VTE in the ARIC Study or with the odds of incident VTE in the LITE sample involving the ARIC Study and the CHS (Table). This conclusion was unchanged after the restricted cubic spline method was used in the ARIC Study or deciles of the distribution were used in the LITE Study. There also was no association with multivariate adjustment for age, race, sex, body mass index, or factor VIIIc.

Combinations of deficiencies have been reported to greatly increase VTE risk.\textsuperscript{23,24} However, these combinations in relation to protein C and antithrombin are relatively rare in the general population. In the ARIC Study, only 3 people at baseline had both protein C $<2.0$ mg/L and antithrombin $\leq 65\%$; none of them had VTE events during follow-up. In the LITE Study, no one had this combination of low protein

![Age-Adjusted Relative Risk for Venous Thromboembolism Relative to Protein C of 3.7 mg/L, ARIC](http://atvb.ahajournals.org/)

The distribution of protein C is represented by the bell-shaped curve (and the right scale). The other solid dark line (and the left scale) represents the RRs for various values of protein C based on a cubic spline model. The dotted lines are the 95% CIs for the RRs.
C and low antithrombin, nor did anyone have 1 of these in combination with factor V Leiden.

Discussion

In the prospective population-based ARIC Study of adults (mean age 54 years), the incidence of VTE not due to cancer was increased in participants with low levels of protein C. The risk of VTE was not reduced further by high levels of protein C, consistent with a threshold. For a protein C value <2.0 mg/L, present in 1.1% of the population, VTE incidence was 3.5 per 1000 person-years; in other words, it was 3.36 times (95% CI 1.2 to 9.1) the VTE rate of those with higher protein C values. This incidence rate of 3.5 per 1000 person-years is approximately the same as the incidence rates reported by other studies.8,9 Our RR estimate is similar to RR estimates from population-based case-control studies with similar ages: RR 3.1 (95% CI 1.4 to 7.0) in the Leiden Thrombophilia Study,2 which had a mean age of 47, and RR 2.9 (95% CI 1.1 to 8.1) in an Oxford study of 45- to 64-year-old women.25 Thus, population-based studies suggest a 3-fold increased risk of VTE that is based on a single measurement of low protein C. This is half the RR of VTE for DNA-confirmed protein C deficiency.2 On the basis of an observed prevalence of low protein C of 1.1% and an RR of 3.36, ~2.5% of VTE in this ARIC sample might be due to low protein C. This figure is similar to Rosendaal’s estimate14 of the population attributable risk of 2% for protein C deficiency.

We found no association between low levels of antithrombin and VTE incidence in these subjects aged >45 years. In contrast, the 2 previous population-based case-control studies found low levels of antithrombin to increase VTE risk. RR estimates were 2.2 (95% CI 1.0 to 4.7) in the Leiden Thrombophilia Study2 and 3.3 (95% CI 1.2 to 9.7) in the Oxford study.25 One possible reason for this discrepancy is that we undoubtedly had few participants with true antithrombin deficiency in the lowest 1% of the antithrombin distribution, given an estimated prevalence of deficiency of 0.02%7 and the fact that we excluded those with a past history of VTE. Previous case-control studies also have used liberal definitions of antithrombin deficiency that yielded prevalences of 1.9% among controls in the Leiden study and 6% among controls in the Oxford study. Varying the cut point for our definition of deficiency did not change our null result. Another possibility for the lack of association is that our antithrombin assay may have been insufficiently accurate to identify the few truly antithrombin-deficient subjects. A final possibility is that the older age of the LITE sample led to a null finding, but the analysis based on middle-aged ARIC subjects was also null.

It has been pointed out that inherited anticoagulant defects, including factor V Leiden, may cluster together in many hereditary venous thrombosis cases.23,24 Many of these cases manifest VTE early in life. However, our data suggest that the clustering of low protein C and low antithrombin, or these in combination with factor V Leiden, is relatively rare in the general population and among patients with VTE onset after the age of 45 years.

Although this is the first prospective study of VTE incidence in relation to low levels of protein C and antithrombin, it has some limitations. First, low levels were defined arbitrarily as the lowest ~1% of participants. Deficiency was not confirmed by a repeat measurement from a separate blood draw or by DNA analysis, as might be done in clinical practice. Thus, our sample with deficiency or not suffers from some misclassification. Second, protein C and antithrombin had low reliability (ie, high method variance plus intra-individual variance), which would attenuate regression coefficients by ~50%. Thus, because of misclassification, the true RR estimates are undoubtedly higher than reported, and the dose-response relation depicted in the Figure may be underestimated. Third, because deficiencies are relatively rare, few participants had low values, even with our large sample size. Because VTE due to protein C or antithrombin deficiency often occurs early in life, some people with known deficiencies might have chosen at baseline not to take part in the ARIC Study. Finally, our antigen-based protein C assay would have detected only the more common type I, and not type II, deficiency.

We excluded from analysis participants with a baseline history of VTE or anticoagulant therapy, as is appropriate for an incidence cohort. We also excluded participants with cancer. Compared with those not excluded, those excluded had an ~1% higher prevalence of low protein C but a similar prevalence of low antithrombin. To determine whether these exclusions affected our findings, we repeated the analyses without exclusions. The RRs for low protein C were only slightly different from those in the Table: RR 1.43 (95% CI 0.73 to 2.80) for protein C <2.3 mg/L and RR 3.27 (95% CI 1.34 to 7.98) for protein C <2.0 mg/L. The RRs for antithrombin, without exclusions, were virtually identical to those in the Table.

In conclusion, we provide prospective evidence that low protein C levels in adults more than 45 years old is associated with a >3-fold increase in VTE incidence. The rarity of protein C deficiency meant that it accounted for ~2.5% of the VTE events in this cohort. In contrast, we found no relationship between low antithrombin levels and VTE incidence.

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