A deficiency of protein C (PC), antithrombin, or protein S is strongly associated with deep-vein thrombosis in selected patients and their families. However, the strength of the association with venous thrombosis in the general population is unknown. This study was a population-based, patient-control study of 474 consecutive outpatients, aged less than 70 years, with a first, objectively diagnosed, episode of venous thrombosis and without an underlying malignant disease, and 474 healthy controls who matched for age and sex. Relative risks were estimated as matched odds ratios. Based on a single measurement, there were 52 (4.6%) patients with a PC deficiency (PC activity, less than 0.67 U/mL or PC antigen, less than 0.33 U/mL when using coumarins). Among the controls, the frequency was 1.5% (seven subjects). Thus, there was a threefold increase in risk of thrombosis in subjects with PC levels below 0.67 or 0.33 U/mL.

**PROTEIN C, PROTEIN S, and antithrombin are components of physiologically important anticoagulant systems.** A deficiency of these proteins can cause thrombosis due to shortage of coagulation inhibition.

To date, a number of investigators have provided estimates of the prevalence of deficiencies of these three major coagulation inhibitors in selected patient groups or in large populations of healthy individuals. Obviously, the wide range in these observed prevalences is related to the selection of the subjects.

Heterozygosity for protein C deficiency, and also for protein S and antithrombin deficiency, has been associated with a tendency to venous thrombosis in selected patients and their families. The total frequencies of these defects among thrombosis patients have been estimated at 3% to 8%. This view was challenged by Miletich et al in 1987, who, among 5,422 healthy blood donors, identified 79 subjects with low protein C levels, none of whom had a personal or family history of venous thrombotic disease.

The results of the studies in selected groups are probably overstated: in studies that investigated a selected group of patients with repeated thrombosis at a younger age or with a clear familial risk, the association between a protein deficiency and deep-vein thrombosis is likely to be higher than in the average thrombosis patient. In contrast, among healthy blood donors recruited on the basis of absence of previous disease, the relation between a protein deficiency and thrombosis is likely to be underestimated. More importantly, most studies were uncontrolled; i.e., there was no reference group or index group, which is crucial to investigate the association between a deficiency and venous thrombosis. The study by Heijboer et al was the first controlled study that included 277 patients and 138 control subjects. However, this study was not designed to estimate the association between the hereditary deficiency and venous thrombosis, but rather to assess the positive predictive value for the presence of an isolated protein deficiency in patients with recurrent, familial, or juvenile deep-vein thrombosis. In another controlled pedigree study by Allaart et al, it was reported that truly heterozygous protein C-deficient individuals had a ninefold higher thrombosis risk than their nondeficient family members. Nondeficient family members are excellent controls to investigate the etiologic question of the association between a deficiency and thrombosis, as they closely resemble the index group. They are less appropriate, however, to estimate the size of the relative risk when these families are identified by a highly selective referral process. This may lead to an underestimate of the true risk, e.g., when the presence of other, possibly yet unidentified, risk factors in the family contributed to their thrombophilia, or it may lead to an overestimate, when families with an abundance of thrombosis (i.e., with several probands) are selectively diagnosed or referred.

At present, the impact of a deficiency of one of the major coagulation inhibitors as a cause of venous thrombosis in the general population is still unclear. Therefore, we have investigated 474 unselected and consecutive patients, aged less than 70 years, with a first, objectively confirmed episode of deep-vein thrombosis and without underlying malignant disease and compared the plasma levels of protein C, protein S, and antithrombin with those of 474 matched healthy con-

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trol subjects. The study was part of a population-based, case-control study on hereditary venous thrombosis: the Leiden Thrombophilia Study (LETS).

PATIENTS AND METHODS

Study design. Patients were selected from the files of the Anticoagulation Clinics in Leiden, Amsterdam, and Rotterdam, The Netherlands. In The Netherlands, anticoagulation clinics monitor coumarin treatment in virtually all patients with venous thrombosis, with each clinic monitoring for patients in a well-defined geographic area. As all of our patients originated from one of these areas, geography was the first criterion for eligibility. We included all 474 consecutive outcome of deep vein thrombosis that occurred between January 1, 1988, and December 31, 1992 (Leiden area, n = 271); January 1, 1989, and December 31, 1992 (Amsterdam area, n = 90); and January 1, 1990, and June 31, 1992 (Rotterdam area, n = 113). Patients with known malignant disorders were excluded. Information about the inclusion and exclusion criteria was obtained from general practitioners, from discharge records from the hospitals, and from the records of the anticoagulation clinics. Patients on short-term coumarin treatment were seen only after anticoagulant treatment in virtually all patients with venous thrombosis, with each clinic monitoring for patients in a well-defined geographic area.

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Each thrombosis patient was asked to find his own healthy control subject according to the following criteria: same sex, same age (±5 years), no biologic relationship, no history of venous thromboembolism, no use of coumarins for at least 3 months, and no known malignant disorders. Partners of patients were also invited to volunteer as control subjects. When a patient was unable to find a control subject, the first individual from the list of partners who matched for age and sex was asked to join the study; 225 (47%) matched control subjects were partners of other patients. The control subjects and cases were inhabitants of the same geographic areas.

All subjects completed a standard questionnaire that contained questions about hormone use at the time of the venepuncture and about the presence of acquired risk factors in the past, confined to a specific period before the index date (ie, date of the thrombotic event). As acquired risk factors, we considered surgery or hospitalizations in the year preceding the index date, postpartum periods (within 30 days of the index date); and pregnancy at the time of the index date.

Laboratory studies. Blood was collected from the antecubital vein into Monovette tubes (Sarstedt, Nümbrecht, Germany) containing 0.106 mmol/L trisodium citrate. Plasma was prepared by centrifugation for 10 minutes at 2000g at room temperature and stored at -70°C in 1.5 mL-volumes until used. High-molecular-weight DNA was isolated from leukocytes and stored at 4°C.

Protein C activity and antithrombin activity were measured with CoamaTE (Chromogenix, Mölndal, Sweden); factor II activity, with a chromogenic method using S-2238 (Chromogenix) and Echis Carinatus (Sigma Chemical Co, St. Louis, MO) on an ACL 200 (Instrumentation Laboratory, Milan, Italy). Protein C antigen was assessed by electrophoresis assay as described elsewhere. Total protein S was measured by polyclonal enzyme-linked immunosorbent assay (ELISA), and free protein S was measured directly in plasma by ELISA using two monoclonal antibodies specific for free protein S (Asserachrom free Protein S, Stago Diagnostica, Asnieres-sur-Seine, France). The results are expressed in units per milliliter, where 1 U refers to the activity or antigen present in 1 mL pooled normal plasma (so, 1 U/mL = 100%). The activated partial thromboplastin time (APTT), prothrombin time; and thrombin time were measured using Cephotest (Nycomed, Oslo, Norway), Thromborel S (Behringwerke AG, Marburg, Germany), and Thrombin Reagent (Beckman, Miami, FL), respectively, on an Electra 1000 (MLA, Pleasantville, NY).

For the identification of mutations in the protein C gene, we used polymerase chain reaction (PCR) amplification followed by direct sequencing.

To exclude observer bias, the technicians were blinded to the status of the sample, ie, whether it was from a patient or a control subject.

The criteria for the diagnosis of protein deficiencies were plasma levels below the lower limit of normal, as used in our laboratory, combined with normal values of factor II and prothrombin time (to exclude a vitamin K deficiency or coumarin use). The lower limits of normal were 0.67 U/mL for protein C and total protein S and 0.80 U/mL for antithrombin. For the new free protein S assay, a reference range was derived from the results in the healthy control subjects. After logarithmic transformation of the data, the lower limit of normal was 0.57 U/mL (mean minus 1.96 SD; none of the subjects had values outside 3 SD of the mean). For those on stable long-term coumarin therapy, the lower limit of normal for protein C antigen (Laurell) and total protein S antigen was 0.33 U/mL. For the primary analysis, we only used the outcomes of the first measurements. In all subjects with a protein C level below 0.67 (or 0.33) U/mL, DNA analysis was performed. For secondary analysis, all subjects with protein C, total protein S, or antithrombin levels below the cut-off points were seen on a second occasion for re-evaluation.

Analysis and statistics. We calculated relative risks, as estimates of the crude matched odds ratios, for each of the protein deficiencies by simple cross-tabulation. We used Miettinen's test-based 95% confidence limits. The relative risk reflects the thrombosis risk when the plasma level is below a certain limit relative to the risk when it is within the normal range, adjusted for the matching factors of age and sex. As the plasma levels of the inhibitory proteins have a continuum of values instead of binary outcomes, we examined if there was a relation between the plasma levels and thrombosis risk (dose-response) by calculating relative risks over levels of the plasma factor (only for those not on oral anticoagulants).
two controls, a mutation was found (Table 2), which led to a thrombosis risk for abnormally low levels of protein C confirmed by DNA analysis of 6.5 (95% CI, 1.8 to 24). Table 1 also shows the drop in prevalence of a protein C deficiency among patients (from 4.6% to 2.7%) and controls (from 1.5% to 0.4%) when more stringent criteria were used to establish a diagnosis. This resulted in a clear increase of the relative risk for thrombosis (from 3.1 to 6.5).

Table 3 gives the odds ratios for the categories of protein C plasma levels, especially in the range where discrimination between true heterozygotes and normal individuals with low levels is difficult. It shows an inverse relation between the risk of thrombosis and the plasma level of protein C, for levels below the reference category (<0.85 U/mL). Table 2 shows that we found slightly more control subjects among those low at remeasurement, 2:9), and contrasted with the male:female ratio for protein C-deficient (13:16) and antithrombin-deficient (13:11) subjects. The two males with low total protein S levels were both from the patient group. There was no difference in frequency of oral contraceptive use between female patients (67%) and controls (73%) with low protein S levels.

Based on a single free protein S measurement in the 426 patient-control pairs not using oral anticoagulants, the relative risk for thrombosis was 1.6 (95% CI, 0.6 to 4.0). When we used both total and free protein S levels below the lower limit of normal as criteria for the diagnosis of a protein S deficiency, the relative risk became 1.7 (95% CI, 0.4 to 6.9).

Table 4 also gives the risk estimates for antithrombin. Based on one measurement, the relative risk for thrombosis

<table>
<thead>
<tr>
<th>Protein C Strata (U/mL)</th>
<th>No. of Patients</th>
<th>No. of Controls</th>
<th>OR* 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.55</td>
<td>4 (4)</td>
<td>1 (1)</td>
<td>4.0 0.5-36</td>
</tr>
<tr>
<td>0.55-0.65</td>
<td>10 (5)</td>
<td>3 (2)</td>
<td>3.7 1.0-13</td>
</tr>
<tr>
<td>0.65-0.75</td>
<td>16 (ND)</td>
<td>10 (ND)</td>
<td>1.8 0.8-4.2</td>
</tr>
<tr>
<td>0.75-0.85</td>
<td>54 (ND)</td>
<td>44 (ND)</td>
<td>1.3 0.9-2.0</td>
</tr>
<tr>
<td>≥0.85</td>
<td>342 (ND)</td>
<td>368 (ND)</td>
<td>1.01</td>
</tr>
</tbody>
</table>

| Patient-control pairs (n = 426) without coumarin use (first measurements). Data in parentheses indicate number of subjects in whom a mutation was found. Abbreviation: ND, not done. * Test for trend, * P < .01. † Reference category. |
was 2.2 (95% CI, 1.0 to 4.7) and became 5.0 (95% CI, 0.7 to 34) when it was based on two persistently low measurements. There was insufficient variation in plasma levels to perform a dose-response analysis (27 of the 29 subjects with initially lowered values had values between 0.70 and 0.80 U/mL).

The number of subjects in whom at least one acquired risk factor was present in the period before the thrombotic event (or before the index date for the control subjects) is presented in Table 5. It shows that acquired risk factors were equally frequent among thrombosis patients with normal and with abnormal test outcomes for one of the coagulation inhibitors (difference, 3%; 95% CI, -17% to 12%), showing that spontaneous thrombotic episodes are not at all limited to the group with protein deficiencies. Among the healthy controls, acquired risk factors were also frequently present (6%), demonstrating that these acquired states in themselves are insufficient to cause thrombosis.

### DISCUSSION

Among 474 consecutive patients aged less than 70 years with a first, objectively diagnosed episode of venous thrombosis and without an underlying malignancy, functional protein C deficiency was the most frequent coagulation inhibitor deficiency. Protein C deficiency also conferred the highest risk of thrombosis risk according to protein C levels. Among the healthy controls, acquired risk factors were also frequently present (6%), demonstrating that these acquired states in themselves are insufficient to cause thrombosis.

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The prevalence of a protein C deficiency among our unselected outpatients was 2.7% to 4.6%, again depending on the criteria, which is similar to the 3.2% (95% CI, 1.5% to 6.2%) prevalence reported by Heijboer et al but is lower than reported in studies that investigated patients with a history of unusual thrombosis at an early age. Obviously, this relates largely to the method of patient selection in the different groups.

Seven distinct mutations were shared by the 15 subjects in whom a protein C gene abnormality was identified (Table 2). The position-230, R → C replacement was most common and was present in seven patients and one control. The position-169, R → Q substitution recurred twice, whereas the remaining five mutations all occurred once. Three of the mutations resulted in premature stop codons. This leaves no room for doubt regarding the causative relationship between low protein C levels and mutation. The relationship between half-normal functional levels of protein C and also obvious for the position-169, R → Q replacement, which involves the arginine in the cleavage site for thrombin. The position-230, R → C substitution is believed to be the direct cause of the protein C deficiency, as this defect is the most common abnormality in Dutch protein C-deficient subjects. The mutation that leads to the insertion of a valine after amino acid position 264 is novel, and, presently, there are no additional data that support the causative nature of this mutation. Comparison of the homologous amino acid sequences of protein C, factor IX, factor VII, factor X, and prothrombin shows that the mutation is in a stretch of two nonconserved amino acids that are flanked by two invariable amino acids at positions 263 and 266. From this it seems reasonable to hypothesize that the mutation disrupts the correct folding of the protein C molecule. More problematic is the interpretation of the position-1515, G → A transition. This nucleotide replacement occurs 15 nucleotides into intron C. It does not seem to create a new consensus sequence for splicing. Therefore, it is possible that the mutation represents a rare sequence variant without functional consequences. One should also note that we do not know whether the nucleotide change is in the allele that putatively cosegregates with the protein C deficiency. This doubt about the certainty of a protein C deficiency in one of the two control subjects may lead to a twofold error in our estimate of the relative risk (6.5 v 13); ie, the true risk might be twice as high as reported in Table 1.

To date, studies among healthy blood donors and among highly selected patient groups have provided estimates of the prevalence within particular populations. However, such prevalences are insufficient to quantify the relation between a deficiency and thrombosis risk. To answer this question, a comparison between unselected patients and population-based thrombosis-free controls is necessary. The controls in the study of Heijboer et al were individuals suspected of deep-vein thrombosis that could not be confirmed by impedance plethysmography. These individuals do not constitute
a satisfactory sample of the population of thrombosis-free controls, as impedance plethysmography is insensitive in detecting calf-vein thrombosis.\textsuperscript{27} It is, therefore, likely that some of these individuals actually had venous thrombosis of the calf veins, which might have been caused by a protein C deficiency. The result of a 1.4% prevalence of a protein C deficiency among the controls, defined as at least two measurements below the lower limit of normal, might, therefore, have been an overestimate. This is supported by the present results; we found a twofold lower prevalence (0.8%) with persistent lowered protein C levels among the controls (two measurements on fresh blood samples).

To our surprise, we found that lowered total protein S levels were not associated with venous thrombosis. By using lower cut-off points, we consistently found slightly more control subjects than patients with lower values (data not shown). Even among those who repeatedly had low levels, controls were overrepresented. The male:female (2:17) ratio was also unexpected for an autosomal inherited disorder. Therefore, we investigated, by multiple linear regression analysis, the influence of oral contraceptives on the protein S level; among the female controls, the age-adjusted reduction in protein S levels was on average 0.09 U/mL for pill users. However, this difference in protein S levels does not explain our result, as the number of women with low protein S levels who were using oral contraceptives was approximately similar for patients and controls. It does show that different cut-off points for protein S antigen levels are required for males and females, if not also for pill users and non-pill users. Nevertheless, even with a separate analysis for men and women and the strictest cut-off point for nonanticoagulated subjects, no clear thrombosis risk emerged (females, 0 cases and two controls ≥0.55 U/mL; no males with levels ≥0.55 U/mL). Our result is in disagreement with many reports of protein S-deficient kindreds, in which venous thrombosis appears highly prevalent and is clearly related to the deficiency.\textsuperscript{24-26} One possibility might be that protein S deficiency is so rare that we found only a few hereditary deficiencies. This explanation seems unsatisfactory, as we found two control subjects who had persistent levels at 0.50 U/mL; ie, were very likely heterozygous. This renders an extremely low prevalence of the deficiency in the general population unlikely. We, therefore, believe that the possibility that the previous findings in families are the result of cosegregating additional genetic defects should be seriously considered. The possibility that protein S is not a clear risk factor for deep-vein thrombosis in unselected consecutive patients is further supported by the finding that, in systems of purified proteins, protein S has little cofactor activity to activated protein C.\textsuperscript{27,28} The data on the free protein S assay showed also no clear relation between a deficiency and thrombosis, although a positive effect cannot be excluded.

The prevalences of low levels of antithrombin are in agreement with the recent literature. Heijboer et al\textsuperscript{7} reported a strikingly similar prevalence for repeatedly low levels of antithrombin of 1.1% among unselected patients with thrombosis. A relative risk could not be re-calculated from that report, as all 138 control subjects had normal antithrombin levels. In the present study, the relative risk varied from 2.2 to 5.0, depending on the applied criteria. The prevalence of antithrombin deficiency among the controls was 1.9% (one measurement) or 0.2% (two repeated measurements), which is close to the recently reported prevalences in healthy blood donors.\textsuperscript{9,29} These findings indicate that antithrombin deficiency is a very rare disease, and this probably explains why our relative risk estimate of 5.0 could not reach statistical significance.

The control subjects were selected by the patients. We do not believe that this practice may have biased our findings on these hereditary abnormalities in the coagulation system, as it is difficult to imagine that patients choose their friends or partners on the basis of unknown plasma levels of anticoagulant proteins.

This population-based, case-control study confirms that, among unselected patients, the prevalence of a (hereditary) protein C deficiency and of lowered protein S and antithrombin plasma levels is relatively low compared with the 20% prevalence of activated protein C-resistant (factor V Leiden) among unselected patients.\textsuperscript{30,31} Although the clinical burden is, therefore, limited, protein C deficiency clearly confers a threefold to almost sevenfold increase in risk of thrombosis.

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REFERENCES

11. Broekmans AW, Veltkamp JJ, Bertina RM: Congenital pro-