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The Netherlands

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Rosendaal, F.R.

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The Leiden Thrombophilia Study (LETS)

Felix J.M. van der Meer[1], T. Koster[2], J.P. Vandenbroucke[2], E. Briët[1] and Frits R. Rosendaal[1,2]

[1]Department of Hematology; [2]Department of Clinical Epidemiology, Hemostasis and Thrombosis Research Center, Academic Hospital Leiden, The Netherlands

Rationale for the Study

Our study grew from an attempt to solve a controversy. In 1987 protein C antigen level was assessed in a large cohort of healthy blood donors (1). Subjects in this cohort who were heterozygous for protein C deficiency did not suffer from venous thromboembolism. Family studies in four of the protein C deficient individuals showed autosomal inheritance of the defect. These findings seemed in contrast with previous reports in which an association between protein C deficiency and an increased incidence of venous thromboembolism was found (2–4). The way in which patients and normal subjects were selected for these studies was thought to play a major factor in the explanation of the differences that were found. In the first reports (2–4) selected patients from families with a high frequency of venous thromboembolism were investigated. This may have resulted in a too high estimate of the risk of disease. In contrast, Miletich et al. (1) investigated a normal population, which does not lead to an estimate of the risk of disease.

To solve the problem of the association of protein C deficiency with venous thrombotic disease the Leiden Thrombophilia Study (LETS) was started. It was designed as a large case-control study aimed at investigating currently known and future risk factors for the development of venous thromboembolism. In this way the role of a deficiency of protein C, protein S or antithrombin and of APC (activated protein C) resistance, elevated von Willebrand factor and factor VIII:c levels, hyperhomocysteinemia, and a recently detected mutation in the gene of factor II in relation to the risk of venous thromboembolism could be established. Some of these had been in mind before the study, others became important during the study. One of the major contributions of the LETS to the field of thrombophilia has been the assessment of APC resistance in conjunction with the factor V Leiden mutation as risk factor for venous thrombosis.

Correspondence to: Felix J.M. van der Meer, Hemostasis and Thrombosis Research Center, Department of Hematology, C2-R, Academic Hospital Leiden, P.O. Box 9600, 2300 RC Leiden, The Netherlands, Tel. +31 (71) 526-2261; Fax +31 (71) 526-6755; e-mail: F.J.M.van_der_Meer@clinhematology.medfac.leidenuniv.nl

Study Design (5)

LETS was designed to assess the importance at the population level of various risk factors for thrombosis, many of which had been identified by family studies. Generally, two approaches are possible: cohort studies and case-control studies. In the former, patients with and without a risk factor are followed over time, and absolute risks (probability of disease, incidence) and relative risks (incidence rate ratio) can be calculated. In a case-control study, patients with thrombosis and healthy controls are included, and relative risks (odds ratios) can be calculated. Due to the rarity of the genetic risk factors for thrombosis, a follow-up study of a truly unselected sample from the population was considered unfeasible, and the case-control design was chosen. A case-control design is perfectly adapted for the study of genetic influences, since one cannot imagine that cases or controls could be in any way selected, except because of the disease that is studied. In contrast to previous studies, rigid criteria were applied to minimize misclassification, minimize selection bias, and maximize power and reliability of the study. This included setting up a system to identify all consecutive thrombotic events, and to only include patients in whom the diagnosis was objectively confirmed.

The organization of anticoagulant monitoring in the Netherlands was important to achieve these goals. In the Netherlands anticoagulation clinics (thrombosis services) are regionally organized. In this way all patients living in a certain area are monitored for their oral anticoagulant therapy by the same anticoagulation clinic, independent of the hospital they were admitted to or the specialist or general practitioner who started the treatment. Patients participating in LETS were recruited from three thrombosis services in the Netherlands: Leiden, Amsterdam and Rotterdam. A total of 474 (90% of eligible) consecutive outpatients younger than 70 years and without a known malignant disorder, with a diagnosis of a first, objectively confirmed, deep vein thrombosis were included (Leiden area: $n=271$, Amsterdam area: $n=90$, Rotterdam area: $n=113$). Information about inclusion and exclusion criteria was obtained from general practitioners, from discharge records from the hospitals and from the records of the thrombosis services. Preferably, patients were seen after discontinuation of oral anticoagulant therapy for at least three months. Forty-eight (10%) of the 474 patients were on long-term

oral anticoagulant therapy and were not allowed to interrupt their medication for various reasons.

Each patient was asked to find a healthy control subject according to the following criteria: same sex, the same age (±5 years), no biologic relationship, no history of venous thromboembolism, no use of coumarin-derivatives 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.

All subjects completed a standard questionnaire about the presence of acquired risk factors in a specific period prior to the index date, i.e., the date of the thrombotic event. Blood was collected and processed in the standard fashion. High-molecular-weight DNA was isolated from leucocytes and stored at 4°C.

Analysis and Statistics

Relative risks were calculated as estimates of the matched odds ratio. The relative risk reflects the thrombosis risk for a specific (category of a) risk factor relative to a reference category (e.g., risk factor absent or lowest quartile).

Results

The mean age for patients and controls was 47 years (range 16–70 for patients, 16–73 for controls). The male/female ratio among patients and controls was 3/4.

Protein C deficiency (5)

Lower limit of normal for protein C activity was set at 0.67 U/ml. For patients on stable long-term oral anticoagulant therapy protein C antigen was assessed by electroimmunoassay (lower limit of normal 0.33 U/ml)[3]. When one measurement of the protein C level was taken into account (Table 1), the relative risk for the development of venous thrombosis was 3.1 (95% CI 1.7–7.0). After repeated testing the frequency of the defect was lower both in patients and controls, and the relative risk became 3.8. When the presence of a mutation was taken as a criterion for heterozygozity for protein C deficiency, the relative risk became 6.5. For the categories of plasma protein C activity levels, Table 2 shows an inverse relation between the plasma level and the odds ratio for the development of venous thrombosis. Depending on the applied criteria the prevalence of protein C deficiency in the control group was between 1.5 and 0.4%. These figures are in close accordance with those reported by Miletich (1). In this setting with patients and controls we found a clearly increased risk of venous thrombosis in persons who are heterozygous for the deficiency. The risk increased with lower protein C activity levels. When stringent diagnostic criteria were used the relative risk became 6.5, which is probably the most reliable estimate of the relative risk of thrombosis associated with hereditary protein C deficiency.

Table 1 Comparison of prevalence and thrombosis risk in 474 patients and 474 control subjects for several definitions for a protein C deficiency

Definition of protein C deficiency	Patients (%)	Controls (%)	OR[a,b]	95% CI
1st measurement <0.67 (0.33[c]) U/ml	22 (4.6)	7 (1.5)	3.1	1.7–7.0
2st+2nd measurement <0.67 (0.33[c]) U/ml	15 (3.1)	4 (0.8)	3.8	1.3–10
1st measurement <0.67 (0.33[c]) U/ml and the presence of a mutation	13 (2.7)	2 (0.4)	6.5	1.8–24

[a]Denotes age- and sex-matched odds ratio.
[b]All discordant patient-control pairs.
[c]Lower limit of normal for those on coumarin treatment.

Table 2 Thrombosis risk for categories of protein C activity levels[a]

Protein C strata (U/ml)	Patients[b]	Controls[b]	OR[c]	95% CI
<0.55	4 (4)	1 (1)	4.0	0.5–36
0.55–0.65	10 (5)	3 (1)	3.7	1.0–13
0.65–0.75	16 (nd)	10 (nd)	1.8	0.8–4.2
0.75–0.83	54 (nd)	44 (nd)	1.3	0.9–2.0
≥0.85	342 (nd)	368 (nd)	1.0[d]	

[a]n=426 patient-control pairs without coumarin use (first measurement).
[b]Number of persons in whom a mutation was found (nd, not done).
[c]Test for trend, $p<0.01$.
[d]Reference category.

Protein S deficiency (5)

The lower limit of normal for total protein S was set at 0.67 U/ml (0.33 U/ml for subjects on coumarin treatment) and for free protein S at 0.57 U/ml. Slightly more control subjects than patients were found with initial and persistent low total protein S levels (Table 3). Thus, no association between low total protein S levels and thrombosis risk could be detected. This striking result could not be explained by the use of oral contraceptives. There was no difference in the use of oral contraceptives between the female patients (67%) and controls (73%) with low protein S levels. Using a single free protein S assessment in the 426 patient-control pairs that did not use oral anticoagulants, a relative risk for thrombosis of 1.6 was found (95% CI 0.6–4.0). When both total and free protein S levels below the lower limit of normal were used as criteria for protein S deficiency the relative risk became 1.7 (95% CI 0.4–6.9). These results are in contrast to reports in the literature where venous thrombosis appears highly prevalent in kindreds with protein S deficiency (6–8). Since we found two control subjects with a persistent low protein S level at 0.50 U/ml, a very low prevalence of protein S deficiency in the population is not a likely explanation. The possibility that the previous findings in families are at least partly the result

Table 3 Prevalence and thrombosis risk in thrombosis patients and control subjects for protein S and antithrombin deficiency

Definition of a deficiency	Patients (%)	Controls (%)	OR (95% CI)
Total protein S:			
1st measurement <0.67 (0.33[a]) U/ml	8 (1.7)	11 (2.3)	0.7[b] (0.3–1.8)
1st+2nd measurement <0.67 (0.33[a]) U/ml	5 (1.1)	6 (1.3)	0.8[c] (0.2–3.0)
Free protein S[d]:			
1st measurement <0.57 U/ml	13 (3.1)	9 (2.1)	1.6[e] (0.6–4.0)
Total and free protein S[d]:			
Both below lower limits of normal	5 (1.2)	3 (0.7)	1.7[e] (0.4–6.9)
Antithrombin:			
1st measurement <0.80 U/ml	20 (4.2)	9 (1.9)	2.2[e] (1.0–4.7)
1st+2nd measurement <0.80 U/ml	5 (1.1)	1 (0.2)	5.0[e] (0.7–34)

[a]Lower limit of normal for those on coumarin treatment.
[b]Seventeen discordant patient-control pairs, in 7 of which the patient had a lowered protein S level and the control subject did not.
[c]Nine discordant patient-control pairs, in four of which the patient had a lowered protein S level and the control did not.
[d]$n=426$ patient-control pairs not using oral anticoagulants.
[e]All discordant patient-control pairs.

of co-segregating additional genetic defects should be considered seriously.

Antithrombin Deficiency (5)

The lower limit of normal was 0.80 U/ml. Based on one measurement the relative risk on thrombosis for antithrombin deficiency was 2.2 (95% CI 1.0–4.7). This figure became 5.0 (95% CI 0.7–34) when it was based on two consistently low measurements (Table 3).

The prevalence of low antithrombin levels is in agreement with the literature, both for patients with thrombosis as for normal subjects (9,10). It can be concluded that antithrombin deficiency is a rare disease, which probably explains why the relative risk estimate of 5.0 in our study did not reach statistical significance.

APC Resistance (11)

APC resistance was measured as the ratio (APC sensitivity ratio) of two activated partial thromboplastin times (Cephotest, Nycomed Pharma, Oslo, Norway), one in the presence and one in the absence of APC. Because low levels of prothrombin, factor X, or both (<0.5 U/ml) increase the APC-sensitivity ratio the test cannot be used in patients on coumarin therapy.

From the total series of patients the first 345 consecutive patients were included in this part of the study. Forty-two patients were on oral anticoagulant therapy and two patients had an initially prolonged APTT consistent with a lupus anticoagulant. After exclusion of these 44 subjects the study population consisted of 301 patient-control pairs. The lower limit of normal was assessed at 2.17. Sixty-four (21%) of the patients and 14 (5%) of the control subjects showed a APC-sensitivity ratio of less than 2.17. The relative risk of venous thrombosis associated with the presence of APC resistance was 6.6 (95% CI 3.6–12.0). In Table 4 it can be seen that the risk of thrombosis increased with a decreasing APC-sensitivity ratio.

Table 4 Thrombosis risk for strata of APC-sensitivity ratios

APC-sensitivity ratio	Patients	Controls	OR[a]	95% CI
≥2.5	163	220	1	
2.0–2.5	84	72	1.6	1.1–2.4
1.5–2.0	36	7	7.4	3.0–18
<1.5	18	2	12.0	2.7–56

[a]Matched (crude) odds ratio; adjustment for VIII:c, protein C, or protein S concentrations or fibrinogen did not effect these results. Test for trend: $p<0.001$.

The relatively high prevalence in the normal population (5%) and the relative risk of nearly 7 for the development of venous thrombosis makes APC resistance the most prevalent hereditary risk factor for venous thrombosis known today.

Factor V Leiden

Resistance to APC was found to be nearly always caused by a guanine to adenine substitution at nucleotide 1,691 in the gene of blood coagulation factor V (12). This mutation predicts the replacement of arginine at position 506 by glutamine (FV Q506 or Factor V Leiden). The 64 patients and their controls of the LETS study which were found to have APC resistance were screened for the presence of the mutation (13). Of the 70 subjects with APC resistance (64 patients and 6 controls) 56 (53 patients and 3 controls) were found to carry the mutation. Six of these (all patients) were homozygous for the defect. In the 14 subjects in which no mutation was detected, APC resistance was only marginally abnormal. None of the 58 subjects without APC resistance carried the mutation.

For patients homozygous for the factor V Leiden mutation a relative risk of venous thrombosis of 80 was calculated: because no homozygous individuals were found among the controls, the relative risk was estimated assuming Hardy-Weinberg equilibrium to calculate the expected number of homozygous individuals in the general population (13).

Oral contraceptives and the risk of venous thrombosis. The use of oral contraceptive drugs increased the risk of venous thrombosis 3.8-fold (95% CI 2.4–6.0) (14). It was found that, in comparison to women not using the oral contraceptive pill and without the factor V Leiden mutation, the risk of venous thrombosis was 28.5 times higher in women with the mutation and using the pill (14). The use of pills containing the third generation progestagen desogestrel led to a relative risk of venous thrombosis of

8.7 in comparison to between 2.2 and 3.8 for anticonceptive pills containing the other types of progestagens (15). In a direct comparison between the oral contraceptives containing desogestrel and 30 μg oestradiol and contraceptives containing all other types of progestagens combined, the age-adjusted relative risk was 2.5 (95% CI 1.2–5.2) (15).

Blood group, von Willebrand factor and factor VIII:c.

We investigated the putative role of blood groups, von Willebrand factor and coagulation factor VIII (Factor VIII:c) as risk factor for the development of venous thrombosis (16). A two-fold increase in the risk of venous thrombosis for subjects with blood group non-O was found in comparison to those with blood group O. Higher levels of vWF and of factor VIII:c were associated with an increased risk of thrombosis. After correction for the influence of the other factors only factor VIII:c level remained as independent risk factor (Table 5). It is clear from Table 5 that the level of factor VIII:c is an important risk factor for the development of venous thrombosis: in comparison to a level of FVIII:c < 100 IU/dl a level of more than 150 IU/dl had a relative risk of 4.8. This high risk stratum (factor VIII:c ≥ 150 IU/dl) comprised 25% of the patients and 11% of the controls.

Hyperhomocysteinemia (17)

In the subgroup of patients and controls who were seen at the Leiden Anticoagulation Clinic, the total plasma homocysteine level was assessed. Hyperhomocysteinemia was defined as a homocysteine level above the 95th percentile in the control group (18.5 μmol/L). Of the 269 patients 28 (10%) had elevated plasma homocysteine levels as compared with 13 of the controls [matched odds ratio 2.5 (95% CI 1.2–5.2)]. The association between elevated homocysteine levels and thrombosis increased with age and was stronger among women than among men.

Table 5. Adjusted thrombosis risk for blood group and for categories of vWF and factor VIII

Risk factor		Odds ratio$_{adjusted}$	95% CI
Blood group (non-O vs O)[a]		1.5	1.0–2.2
vWF:Ag strata[b] (IU/dl)	< 100	1	
	100–125	1.1	0.7–1.9
	125–150	1.3	0.7–2.2
	≥ 150	1.2	0.6–2.1
FVIII:c strata[c] (IU/dl)	< 100	1	
	100–125	2.3	1.3–3.8
	125–150	3.0	1.6–5.7
	≥ 150	4.8	2.3–10

[a]Adjustment for vWF:Ag and FVIII:c levels.
[b]Adjustment for blood group and FVIII:c levels.
[c]Adjustment for blood group and vWF:Ag levels.

Table 6. Frequencies and thrombotic risk for the 20210 G/A genotypes in the prothrombin gene

Genotype (nt20210)	Patients (%)	Controls (%)	OR[a]	95% CI
GG	442 (93.8)	463 (97.7)	1.0[b]	
AG	29 (6.2)	11 (2.3)	2.8	1.4–5.6
AA	—	—		

[a]Adjustment for age and sex, current pill use (yes/no), body mass index, in menopause (yes/no) and smoking (yes/no) did not affect these results.
[b]Reference category.

Table 7. Thrombosis risk and prothrombin level

Prothrombin activity (U/ml)	Patients[a] (n=426) (%)	Controls (n=474) (%)	Total (n=900) (%)	OR[b]	95% CI
< 0.95	85 (20)	134 (28)	219 (24)	1.0[c]	
0.95–1.04	107 (25)	125 (26)	232 (26)	1.3	0.9–2.0
1.05–1.15	102 (24)	118 (25)	220 (24)	1.4	0.9–2.0
> 1.15	132 (31)	97 (20)	229 (25)	2.1	1.5–3.1

[a]Patients on coumarin treatment excluded (n=48).
[b]Test for trend, P<0.001.
[c]Reference category.

Factor II (G20210A)

Very recently, a new risk factor for venous thrombosis was found (18). A G to A transition at nucleotide position 20210 in the 3' untranslated region of the prothrombin gene was found to be associated with increased levels of prothrombin activity and with the occurrence of venous thrombosis. In the LETS study population the prevalence of carriers of the 20210 A allele was 2.3% in the healthy controls and 6.2% in the patients (18). This indicates a relative risk of thrombosis of 2.8 (Table 6). In a series of selected patients with a personal and family history of venous thrombosis the mutation was found in 18% (18). Also, an association could be shown between prothrombin levels and the risk of thrombosis (Table 7). Although it is unclear how the mutation of G20210 A leads to higher prothrombin levels, these data suggest that the prothrombin level is the effector of the thrombosis risk. Possibly other factors can also be involved in elevated prothrombin levels.

Conclusions

The relative risk of venous thrombosis in the population as a whole was assessed for a number of hereditary abnormalities by a population based case-control study. The application of rigorous methodology, i.e., the inclusion of consecutive patients, and the inclusion only of objectively confirmed thrombosis, made it possible to reliably assess the importance of a variety of risk factors for thrombosis in the general population. A case-control study is eminently suited for the study of (rare) genetic risk factors since one cannot imagine that cases and controls could be selected

genetically, except because of the presence of the disease. Moreover, genetic risk factors can still be measured "after" the event, in contrast to many lifestyle risk factors. Only for frequent genetic risk factors (like the factor V Leiden mutation) would a foolw-up study be feasible. Even then, a case-control approach might be more cost-efficient.

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