of an impact of ivermectin on OND in follow-up periods of 1–3 years, but those studies had much smaller sample sizes than our trial.

We must be cautious in extrapolating our findings to other onchocercal communities. The likely impact of ivermectin on OND (all causes) will depend on the proportion of OND in those communities that is due to onchocerciasis. DEC was widely used in the area where our trial took place, and though we found no evidence that the rate ratio for ivermectin versus placebo varied with previous consumption of DEC, we have no reliable, systematic data on the consumption of DEC during the trial; the effect of ivermectin on OND due to onchocerciasis may vary with the extent of continuing DEC consumption.

Unpublished data from our trial suggest that OND is an important pathway to blindness due to guinea savannah onchocerciasis. Regular treatment with ivermectin may substantially reduce the risk of onchocercal blindness among individuals living in the guinea savannah with microfilarial loads above 10 mf/mg.

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Increased risk of venous thrombosis in carriers of hereditary protein C deficiency defect

C. F. ALLAART SWIBERTUS R. POORT FRITS R. ROSENDAAL
PIETER H. REITSMA ROGIER M. BERTINA ERNEST BRIËT

The relevance of heterozygosity for hereditary protein C deficiency as a risk factor for venous thrombosis has been disputed because heterozygotes without symptoms have been identified among blood donors and relatives of homozygotes. As a result, clinicians do not know whether to offer prophylaxis or not. We have compared thrombosis-free survival in 161 heterozygous and normal members of the families of 24 heterozygotes for protein C deficiency referred from several centres in the Netherlands and with a history of symptoms. We studied the influence of heterozygosity and of putative additional risk factors on the occurrence of thrombotic events noted when a medical history was taken. Protein C activities were measured but a diagnosis of heterozygosity was based on the presence of the specific mutation in one of the protein C genes identified in the proband of the family.

We found a significant difference in the thrombosis-free survival of the 77 heterozygotes and 84 normals. by age 45, 50% of heterozygotes and 10% of normal relatives can be expected to have had a manifestation of venous thromboembolism. The presence of such a mutation was clearly associated with an increased risk of venous thrombotic events. Thrombotic events occurred more often in years in which the patient had been immobile for more than a week or had had surgery. Other putative risk factors showed no significant effect in the incidence of thrombotic events. About 50% of all first episodes and 65% of recurrences of venous thromboembolism in the heterozygotes were spontaneous—ie, there was no predisposing event such as surgery or pregnancy. There was no increased risk for arterial occlusions in heterozygotes.

We conclude that members of the family of a symptomatic heterozygote proband who are heterozygous for the mutation in the protein C gene have an increased risk of venous thrombotic events compared with their normal family members. For such individuals prophylactic anticoagulation should be considered; the decision will need to be taken on an individual basis.

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ADDRESSES **Thrombosis and Haemostasis Research Unit, Department of Hematology** (C F Allaart, MD, S R Poort, PhD, P H Reitsma, PhD, R M Bertina, PhD, E Briet, MD, F R Rosendaal, MD) **and Department of Clinical Epidemiology** (F R Rosendaal), **University Hospital Leiden, Leiden, Netherlands.** Correspondence to Dr C F Allaart, Thrombosis and Haemostasis Research Unit, University Hospital Leiden, PO Box 9600, 2300 RC, Leiden, Netherlands

Introduction

Protein C is a vitamin-K-dependent glycoprotein that is the zymogen of one of the main inhibitors of the coagulation system.[1] After activation by the thrombin-thrombomodulin complex, activated protein C inactivates factors Va and VIIIa[2,3] in the presence of protein S, calcium ions, and phospholipids.[4] Since protein C has anticoagulant properties it is not surprising that heterozygosity for hereditary protein C deficiency has been associated with a tendency to venous thrombosis.[6-10] In 1983 the first patient with homozygous protein C deficiency was reported.[11] Surprisingly, his heterozygous family members were not thrombosis prone, and subsequent reports confirmed this in other families.[12-15] In 1987 Miletich et al identified 79 heterozygotes for protein C deficiency among 5422 healthy blood donors. None had experienced a thrombotic event. In 4 of them the hereditary nature of the deficiency was confirmed.[16] In the light of these findings, a role for heterozygous protein C deficiency as a risk factor in venous thrombosis has been disputed.

Clinicians want to know if prophylactic measures are advisable for patients with protein C deficiency and their relatives. Before this question can be answered the risk for thrombosis in these individuals needs to be assessed. We have studied 24 families with hereditary protein C deficiency in which at least one person had had thrombosis. The diagnosis of the deficiency was based on the presence or absence of specific point mutations in protein C genes. We then calculated the thrombosis-free survival of the heterozygotes and the genetically normal relatives.

Patients and methods

Patients and families

The study was approved by the University Hospital, Leiden medical ethics committee and informed consent was obtained from all participants. When the study began 80 Dutch probands with venous thrombosis and protein C deficiency were known to this centre. The diagnosis was based on the repeated finding of protein C antigen levels below 0·65 U/ml for patients not on oral anticoagulants or below 0·33 U/ml for those on stable coumarin therapy.[6] From these 80 probands, 25 were randomly selected for DNA analysis. In 24 probands a point mutation was identified in one protein C gene (table 1).[17] All siblings and children (over 16 years of age) and parents of the proband plus siblings of the deficient parent were invited to take part in the study. Of the 175 eligible individuals 161 (92%) did take part; 7 non-participants lived abroad and 7 others did not take part because of poor health (3) or for reasons unknown (4). 18 family members who would have been eligible had died before the study began. 6 of these 18 were reported to have had thrombosis, and pedigrees suggested that 3 of these 6 were obligatory heterozygotes for the deficiency. The genotype of the others is unknown. 64 of the 161 participating family members had already been tested for protein C deficiency and abnormally low antigen levels had been found in 35. They knew this earlier result but we did not. DNA analysis had not been done previously.

All 161 participants were interviewed by one physician (C. F. A.), who took a medical history with emphasis on manifestations of deep venous thrombosis (DVT), pulmonary embolism, and superficial thrombophlebitis. Age at each such manifestation was noted, together with possible predisposing circumstances, method of diagnosis, and treatment. Participants were also asked about potential risk factors for the venous thrombosis—ie, surgery, immobilisation, pregnancy and childbirth, cardiac and liver disease, malignancy, varicosities, smoking, and oral contraceptives—and questions were asked with respect to the manifestation of arterial occlusive disease.

Laboratory methods

Blood samples were collected from the antecubital vein in 1/10 volume of 0·11 mmol/L trisodium citrate. Plasma was prepared by centrifugation for 10 min at 2000 *g* at 10°C and stored at −70°C. High-molecular-weight DNA was isolated from leucocytes and stored at 4°C.

TABLE I—MUTATIONS FOUND IN 24 FAMILIES WITH HEREDITARY PROTEIN C DEFICIENCY TYPE I

Mutation	Exon	No of families	No of heterozygotes (participants)
^{230}Arg→Cys	9	7*	16 (37)
^{132}Gln→stop	6	5	10 (35)
^{76}Phe→Leu	5	9	10 (21)
^{306}Arg→stop	9	2†	10 (18)
^{105}Cys→Tyr	6	2	10 (17)
^{178}Arg→Trp	7	1	3 (10)
^{403}Ile→Met	9	1	7 (8)
^{256}Asn→Asp	9	1	4 (6)
^{292}Gly→Ser	9	1	4 (5)
3222 G→T	5‡	1	3 (4)

*Two families related through common ancestors in 1820.
†Related through common ancestors in 1880.
‡Donor splice site 3′ side of exon 5.

Protein C activity was measured with Coatest (Kabi Diagnostica, Stockholm) on an ACL 300 (Instrumentation Laboratories, Milan). Pooled normal plasma from 67 healthy volunteers served as a reference. Protein C activity is expressed in U/mL, where 1 U refers to the activity in 1 mL pooled normal plasma.

We used polymerase chain amplification (PCR)[18] to detect alterations in the coding sequence of the protein C gene in the probands. In family members mutations were identified as altered digestion patern for particular restriction enzymes or by direct sequencing.[17] The identification of heterozygotes and normals was done by two molecular biologists (S. R. P., P. H. R) who did not know the protein C measurements or the medical history.

Statistics

Laboratory data are expressed as mean (SD) with comparisons by Student t test. We constructed thrombosis-free survival curves by the Kaplan and Meier method,[19] comparing them by a logrank test (chi-square distribution, one degree of freedom). Confidence intervals (CI) for the thrombosis-free survival rates were calculated on a binomial distribution.

The incidence of first thrombotic events in heterozygotes and normals was calculated by counting patient-years of observation and dividing the number of events in each group by the sum of observation-years of all the individuals in the group. Similarly, incidence rate ratios for surgery and immobilisation were calculated as the ratio of the incidence of thrombosis in the years that surgery (or immobilisation) took place and the incidence rate in all other years. CI for incidence or incidence rate ratios were calculated on a Poisson distribution.[20]

We calculated crude (univariate) odds ratios as an approximation of relative risk for several putative risk factors by simple cross-tabulation. These odds ratios reflect the risk when the proposed risk factor is present relative to the risk when it is absent, unadjusted for other factors. We then used a Cox proportional hazards model in a multivariate survival analysis allowing several factors to be adjusted for simultaneously. The hazard ratio is the incidence rate ratio, which is assumed to be constant over time, whereas the baseline hazard is allowed to vary.[21] This hazard ratio may be interpreted as the relative risk associated with each factor, adjusted for all other factors in the model.

Results

Laboratory results

In the twenty-four families ten different mutations were identified, five in more than one family (table 1). Extensive genealogical studies proved a common ancestry in the two ^{306}Arg→stop families and in two of the ^{230}Arg→Cys families[17] but no such connections were found in the other

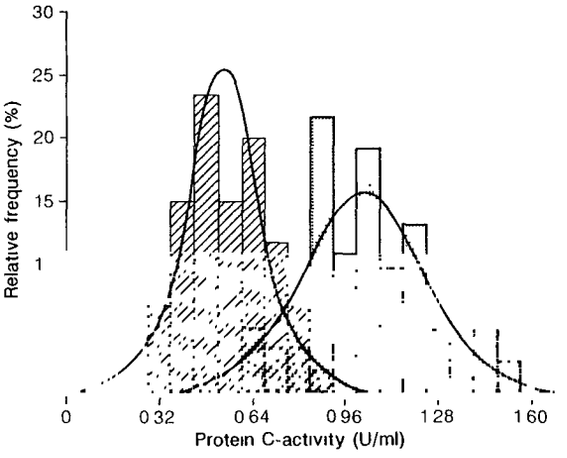

Fig 1—Distribution of plasma protein C activity in heterozygotes and normal relatives.

Concentrations in 60 heterozygotes (diagonal line shading) and 83 normal relatives (dotted shading) not on oral anticoagulant therapy plotted as frequency histogram with intervals of 0·08 U/mL. Curves represent expected distributions.

families. Of the 161 participants, 77 were heterozygous for protein C deficiency (33 men, 44 women; average age 47·2 years, range 18–83), on the basis of mutations in the protein C gene. 84 were normal (38 men, 46 women; average age 45·3 years, range 16–82).

In every family low protein C activity levels co-segregated with the mutation—ie, both had been transmitted together through the generations. However, not all heterozygotes had a low protein C activity, and there was a large overlap in protein C activities measured in 60 heterozygotes and 83 normals not on anticoagulant therapy (fig 1). Fitting a gaussian curve to the frequency distributions suggested that 15% of heterozygotes would not be identified and 5% of normals would be labelled deficient if the diagnosis were to be based on protein C activity only, with the lower limit of normal of 0·65 U/mL. Heterozygotes not on oral anticoagulant therapy and with no symptoms had slightly higher protein C activities (56·0 [13·7] U/mL) than heterozygotes with symptoms (50·8 [13·0] U/mL), but this difference was not significant.

Clinical data

Of the 77 heterozygous relatives of the probands 35 (45%) had experienced one or more venous thrombotic events; 16 had previously been diagnosed as deficient but the other 19 were newly diagnosed heterozygotes. Of 7 heterozygotes previously labelled normal on the basis of protein C antigen levels, 1 reported having had a thrombotic event.

First symptoms were DVT in a leg in 21 heterozygotes and pulmonary embolism in 10. Diagnosis and anticoagulant treatment were based on clinical presentation only in 21 of these 31 events. 4 heterozygotes had superficial thrombophlebitis as a first manifestation and 8 had a combination of deep and superficial thrombosis. The first episode was spontaneous in 17 patients (49%); in 8 the episode followed surgery or trauma, in 7 childbirth (2 caesarean sections), in 2 during pregnancy, and in 1 during a period of immobilisation exceeding 1 week. More than one event had occurred in 18 of the 35 heterozygotes with symptoms. 22 of the 34 recurrences were spontaneous (65%); the others were associated with trauma or surgery (4), immobilisation (2), pregnancy (4), and childbirth (2).

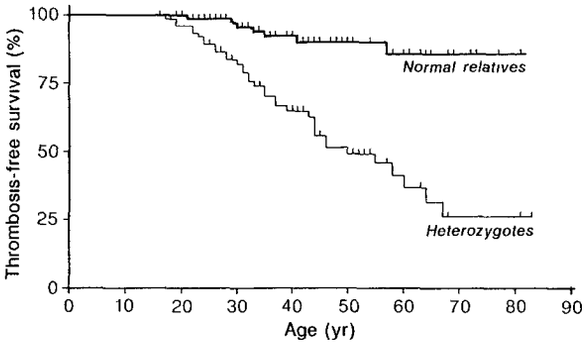

Fig 2—Venous-thrombosis-free survival curves in heterozygotes and normal relatives.

Probability of freedom from venous thrombotic events presented in Kaplan-Meier analysis for 77 heterozygotes and 84 normal relatives. Difference in curves significant ($\chi^2 = 25\ 3$; $p < 0\ 0005$). Steps in curves indicate events; short vertical lines indicate losses to the analysis because of age of survivor.

7 of the 84 normal relatives, in five different families, had manifestations of venous thromboembolism: 3 had DVT in a leg, 2 had pulmonary embolism, and 2 had superficial thrombophlebitis. In 3 of the 5 deep thrombotic events diagnosis and anticoagulant treatment had been based on the clinical presentation only. The first episode was spontaneous in 3, after surgery in 1, childbirth in 2, and prolonged immobilisation during pregnancy in 1. 3 normal relatives had recurrences and of the 13 recurrences 4 were spontaneous and 1 followed surgery and 8 were after childbirth.

The thrombosis-free-survival curves (fig 2) show that at the age of 45 the probability that a heterozygote will be free of venous thrombosis is 0·5 (95% CI 0·31–0·71) while for normal relatives it is 0·9 (95% CI 0·8–1·0). The incidence of first thrombotic events in heterozygotes was 4·1 per 1000 person-years up to age 25 (*vs* 0·5 per 1000 in normal relatives); between 26 and 45 years it was 17·0 per 1000 person-years (*vs* 4·3), and between 46 and 83 years it was 12·9 per 1000 person-years (*vs* 1·7).

The difference in thrombosis-free survival in fig 2 cannot be attributed to differrring frequencies of potential risk factors for thrombosis (table II). The risk of venous thrombosis was strongly influenced by the presence of a mutation in one of the protein C genes (hazard ratio 8·8, 95% CI 3·6–21·5) (table III). The incidence of thrombotic events was greater in the years in which the participants had had an operation (88 per 1000 person-years, compared with 10 per 1000 in years with no surgery; incidence ratio 9·2 [95% CI 5·0–16·8]). It was also greater in years in which they had been immobilised for more than a week (82 per 1000 person-year compared with 11 per 1000 person-years

TABLE II—POTENTIAL RISK FACTORS IN 77 HETEROZYGOTES AND 84 NORMAL RELATIVES

Potential risk factor	No of individuals (no of episodes) in:	
	Heterozygotes	Normal relatives
Female sex	44	46
Surgery/trauma	54 (120)	62 (127)
Immobilisation	35 (52)	33 (55)
Pregnancy and childbirth	34 (87)	32 (119)
Oral contraceptive use*	24	27
Smoking*	36	47
Overweight by > 10 kg	7	10
Varicosities	18	15
Malignancy*	0	1

*Mentioned is number of individuals in whom risk factor was "ever" present

TABLE III—EFFECT OF POTENTIAL RISK FACTORS ON THE OCCURRENCE OF VENOUS THROMBOTIC EVENTS IN ALL 161 PARTICIPANTS

Potential risk factor	Venous thrombotic event (yes/no) in: Exposed	Non-exposed	Crude OR	Hazard ratio* (95% CI)
Heterozygosity	35/42	7/77	9·2	8·8 (3·6–21·5)
Female sex	28/62	14/57	1·8	1·7 (0·4–5·9)
Surgery/trauma†	34/82	8/37	1·9	1·9 (0·8–4·6)
Immobilisation†	21/47	21/72	1·5	1·2 (0·7–2·3)
Pregnancy and childbirth†	24/42	18/77	2·4	0·9 (0·3–2·9)
Oral contraceptive use†	15/36	27/83	1·3	1·3 (0·5–3·2)
Smoking†	20/63	22/56	0·8	1·2 (0·6–2·4)
Overweight > 10 kg	6/11	36/108	1·6	1·7 (0·6–4·5)
Varicosity	15/18	27/101	3·1	1·5 (0·7–3·3)
Malignancy†	0/1	42/118	0	2·7 (0·3–27·9)

*Proportional hazards model; all listed proposed risk factors entered.
†"Ever" had risk factor compared with "never".

in years without immobilisation; incidence rate ratio 7·6 [95% CI 3·7–15·7]). An association between a history of surgery or immobilisation and an increased risk of venous thrombotic events was also found in crude odds ratios and in the proportional hazards model. In the proportional hazards model overweight, female sex, a history of malignancy, and, to a lesser extend, varicosities and a history of oral contraceptive use or cigarette smoking, all appeared to be associated with an increased risk, but for none of these factors was this effect significant (table III).

7 heterozygotes had experienced manifestations of arterial occlusion: 1 had angina pectoris at age 69; 2 had a myocardial infarction at age 50 and 67, 1 had intermittent claudication at 45; 1 had a stroke at 73; 2 had a transient ischaemic attack at 48 and 72. 5 normal relatives had experienced manifestations of arterial occlusion: 1 had angina pectoris at age 60; 1 had a myocardial infarction at 60; 1 had intermittent claudication at 78; and 2 had a transient ischaemic attack at 18 and 50. 35 heterozygotes and 29 normals were over 50. These limited data do not suggest that a heterozygous state for protein C deficiency is associated with an increased risk of arterial occlusion.

Discussion

Eleven years after the first report on hereditary protein C deficiency, the importance of this as a risk factor for venous thrombotic events is still uncertain. In many family reports an association between a thrombotic tendency and protein C deficiency has been established, and in groups of selected patients with venous thrombotic disease the frequency of protein C deficiency was 3–8%.[22-24] It was estimated, from a study in 319 patients treated with oral anticoagulants for venous thrombosis, that the prevalence of hereditary protein C deficiency in the population is 1 in 16 000.[25] However, the identification of symptom-free heterozygotes in a large group of healthy volunteers put the prevalence at between 1 in 200 and 1 in 300.[16]

In our study the participants, being aware of a thrombotic tendency in their families, may have overstated the history of thrombotic events. Overstatement is all the more likely because many of the events had been diagnosed and treated on the basis of clinical presentation only. However, 40 of the 42 first thrombotic events predated the diagnosis of protein C deficiency in the family and the participants did not at the time of interview know whether they carried the defect or not. Any bias, in patients or physicians, will be the same for heterozygotes and for normals.

The heterozygous relatives (parents, siblings, children, aunts, and uncles) of our symptomatic patients with protein C deficiency were at increased risk of a venous thrombotic event. Identification of those at risk is therefore worthwhile. This may be difficult if the diagnosis depends on protein C activity alone because of the overlap between plasma heterozygotes and normals (fig 1). In our study, 13 of the 77 heterozygotes identified by DNA analysis had protein C activity levels that lay within the normal range for our laboratory, and 2 of the 84 normal relatives had a protein C activity just below that range. It is not clear whether the risk of venous thrombosis is related to the level of protein-C activity. There was no difference in activity levels in asymptomatic heterozygotes and in heterozygotes who had had a thrombotic event in the past. Shortly after participating in our study 1 man was admitted to hospital with spontaneous pulmonary embolism. His protein C activity 2 weeks earlier had been normal (76%). He proved to be heterozygous for the ^{230}Arg→Cys mutation present in his family.

The difference in thrombosis-free survival between the heterozygotes and the normal relatives cannot be explained by a difference in potential risk factors for thrombosis. When several such factors were taken into account at the same time it was the presence of a protein C gene mutation that emerged clearly as carrying the highest risk for a venous thrombotic event (table III). Being female or overweight or having a history of malignancy, varicosity, smoking, or the use of oral contraceptives seemed to be associated with some increase in risks but individually these were not significant. In the heterozygotes 9% of all episodes of trauma, surgery, immobilisation and childbirth were complicated by a thrombotic event. In normal relatives this proportion was 4%. These thrombotic complications accounted for 50% or so of all first manifestations and 50% of recurrences. All other thrombotic episodes were "spontaneous".

In a family with hereditary protein C deficiency in which at least one person has had a thrombotic event, prophylaxis must be considered for heterozygotes whether or not they have symptoms. However, the benefits and risks of anticoagulant therapy are difficult to establish in general terms so any advice will have to be given individually.

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Frequency of clonal remission in acute myeloid leukaemia

ROSEMARY E. GALE HELEN WHEADON ANTHONY H. GOLDSTONE
ALAN K. BURNETT DAVID C. LINCH

Analysis of X-chromosome inactivation patterns in females has been used to assess clonality of various tumours and for prenatal diagnosis of X-linked disorders. Studies with these methods in acute myeloid leukaemia suggest that a significant proportion of cases have clonal remissions (ie, persistence of the malignant clone), which may represent return to a preleukaemic state. We therefore analysed X-chromosome inactivation patterns with differential methylation patterns of heterozygotes for three DNA probes, HPRT, PGK, and M27β, in leukaemic patients and normal controls.

As expected, blast cells from 67 of 68 analysable samples (99%) were monoclonal or had a skewed X-inactivation pattern. A skewed pattern in remission was also found in 26 of 77 patients (34%), proportion only slightly greater than control (16/75, 21%). In 7 of 10 patients with a skewed pattern in myeloid cells there was similar skewing in the T cells, which is compatible with the concept of a constitutively skewed X-chromosome inactivation pattern of haemopoietic cells in these patients.

Our study illustrates the difficulty of interpreting clonality in individual tumour samples and emphasises the importance of comparisons with non-malignant tissue of the same cell type from that individual and from normal control populations.

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Introduction

Clinical remission in acute myeloid leukaemia (AML) is the restoration of a normal peripheral blood count with less than 5% blast cells in the bone marrow,[1] which is generally thought to represent the return of normal polyclonal haemopoiesis. However, Powles et al[2] raised the possibility that many cases of remission could be due to differentiation of residual leukaemic cells after chemotherapy, and use of X-linked techniques has shown apparent clonality or oligoclonality of remission granulocytes.[3-5] This observation might represent persistence of the leukaemic clone at an earlier stage in its evolution, when a growth advantage over normal cells is present but cell growth is still regulated and full differentiation occurs.

In females, clonality can be studied by analysing X-chromosome inactivation patterns with techniques that distinguish between alleles on the two X chromosomes. The inactivation of one of the X chromosomes in each cell at an early stage in embryogenesis is a random process called Lyonisation.[6] A population of normal cells will therefore contain a mixture of the two alleles in the ratio in which they are actively expressed. A clone of tumour cells expresses only a single allele, as inherited from the original aberrant cell. The first studies used isoenzymes of glucose-6-phosphate

ADDRESSES **Department of Haematology, University College London Medical School, London WC1E 6HX** (R E Gale, PhD, H Wheadon, BSc, A H Goldstone, FRCPath, Prof D C Linch, FRCP), **and Department of Haematology, University Hospital of Wales, Cardiff, UK** (Prof A K Burnett, FRCPath) Correspondence to Dr R E Gale