COEXISTENCE OF HEREDITARY HOMOCYSTINURIA AND FACTOR V LEIDEN — EFFECT ON THROMBOSIS

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Abstract *Background.* Venous and arterial thromboembolism occurs in only about one third of patients homozygous for homocystinuria, which suggests that other, contributory factors are necessary for the development of thrombosis in these patients. Factor V Leiden, an R506Q mutation in the gene coding for factor V, is the most common cause of familial thrombosis and could be a potentiating factor.

Methods. We determined activated partial-thromboplastin times in the presence and absence of activated protein C and tested for the factor V Leiden mutation in 45 members of seven unrelated consanguineous kindreds in which at least 1 member was homozygous for homocystinuria.

Results. Thrombosis (venous, arterial, or both) occurred in 6 of 11 patients with homocystinuria (age, 0.2 to 8 years). All six also had the factor V Leiden mutation. One patient with prenatally diagnosed homocystinuria who was also heterozygous for factor V Leiden has received warfarin therapy since birth and has not had thrombosis (age, 18 months). Of four patients with homocystinuria who did not have factor V Leiden, none had thrombosis (ages at this writing, 1 to 17 years). Three women who were heterozygous for both homocystinuria and factor V Leiden had recurrent fetal loss and placental infarctions.

Conclusions. Patients with concurrent homocystinuria and factor V Leiden can have an increased risk of thrombosis. Screening for factor V Leiden may be indicated in patients with homocystinuria and their family members. (N Engl J Med 1996;334:763-8.)

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Homocystinuria is caused by deficient activity of one of several enzymes in methionine metabolism, either as a primary defect or as a result of defects in the cytosolic metabolism of cobalamin.[1-4] Hyperhomocysteinemia can also be due to a deficiency of vitamin B_6, folate, or vitamin B_{12}.[5,6] The major clinical features of patients homozygous for homocystinuria are mental retardation, ectopia lentis, skeletal abnormalities, and life-threatening thromboembolic events.[2,3] It is intriguing that thrombosis develops in only about one third of patients with homocystinuria; the reason for this variability, which is found even among siblings, is unknown.[7] The thrombotic episodes, which are likely to occur before the age of 30, include deep-vein thrombosis, pulmonary embolism, and arterial thrombosis involving in particular cerebral, peripheral, and coronary vessels.[2,7]

Hereditary resistance to activated protein C is currently regarded as the most frequent cause of familial thrombosis.[8] Activated protein C is an important physiologic anticoagulant. Generated from protein C on the surface of endothelial cells by the action of thrombomodulin-modified thrombin, it inactivates factors VIIIa and Va.[9] Most cases of resistance to activated protein C stem from a missense mutation in the gene coding for factor V, in which adenine replaces guanine at nucleotide 1691 (G1691A). This change leads to the substitution of glutamine for arginine at position 506 (R506Q), thereby altering the first cleavage site involved in the activation of factor V.[10,11] This mutation, also designated factor V Leiden, has been found in 30 to 60 percent of cases of familial thrombophilia in patients of various ethnic origins and in 3 to 7 percent of healthy people in two white populations.[8,12] Although the risk of thrombosis increases by a factor of 50 to 100 among persons homozygous for factor V Leiden and by a factor of 5 to 10 among heterozygotes,[8,13] many people with the mutant gene may not have signs of thrombosis unless they also have another genetic defect, such as a deficiency of protein C or protein S,[14,15] or unless they are exposed to additional precipitating factors, such as oral contraceptives, pregnancy, or surgery.[8,16]

We investigated whether the coexistence of additional genetic defects or acquired conditions affected the expression of thrombosis in patients with homocystinuria.

METHODS

Family Studies

The study population comprised 45 members of seven unrelated Israeli Arab families in which at least 1 member was homozygous for homocystinuria. Informed consent was obtained from the parents of the patients and from other adult family members. The study was approved by the Human Studies Ethics Committee of Rambam Medical Center. We constructed detailed pedigrees (Fig. 1) and reviewed the medical files and autopsy reports of the patients homozygous for homocystinuria.

Biochemical and Enzymatic Studies

Plasma concentrations of amino acids were determined by ion-exchange chromatography. Free and protein-bound plasma homocysteine concentrations were measured by high-performance liquid chromatography.[17] Methylmalonic acid was quantitated by gas chromatography and mass spectrometry.[18]

Homozygosity for homocystinuria was diagnosed on the basis of clinical features, elevated plasma homocysteine levels (range, 43 to 370 μmol per liter; range in normal subjects, 6 to 19 μmol per liter), and elevated urinary homocysteine levels (150 to 520 μmol per gram of creatinine). The plasma methionine level ranged from 1.5 to 15 mg per deciliter (100 to 980 μmol per liter) in patients with cystathionine β-synthase deficiency and was reduced or normal in patients with methylenetetrahydrofolate reductase deficiency or cobalamin defects.

Human fibroblasts were obtained from skin-punch biopsy speci-

mens from all the patients homozygous for homocystinuria. Fibroblasts were grown in Eagle minimum essential medium (Sigma Chemical, St. Louis) as previously described,[19] and cystathionine β-synthase deficiencies, methylenetetrahydrofolate reductase deficiencies, and cobalamin C/D defects (combined deficiencies of 5′-deoxyadenosylcobalamin and methylcobalamin) were confirmed with slight modifications of previously described methods.[20-24] Similar procedures were used with amniocytes obtained by amniocentesis for prenatal diagnosis.

Measurements of Coagulation

Samples of whole blood (4.5 ml) were collected in Vacutainer tubes containing 0.5 ml of 0.12 M sodium citrate and were centrifuged at $2000 \times g$ for 20 minutes to obtain platelet-poor plasma, which was kept frozen at $-70°C$ until it was tested. The normal values for each test were defined as ranging from -2 SD to $+2$ SD of the mean value in 20 normal subjects. Protein C antigen was determined by enzyme immunoassay with a protein C kit (Asserachrom, Stago, Asnières, France; normal range, 60 to 136 units per deciliter). Protein C activity was determined by a chromogenic method with a protein C kit (Stachrom, Stago). The assay was calibrated with a calibration plasma (Instrumentation Laboratory, Milan, Italy; normal range, 78 to 146 units per deciliter). Antithrombin III activity was determined by a chromogenic method (Antithrombin III Asserachrom kit, Stago; normal range, 78 to 128 units per deciliter). Total protein S antigen was determined with the Asseraplate Protein S kit (Stago; normal range, 70 to 130 units per deciliter). Free protein S was determined by electroimmunoassay of the plasma supernatants after precipitation with polyethylene glycol (Sigma 5000, 25 percent; normal range, 65 to 130 units per deciliter).

Assay for Resistance to Activated Protein C

Resistance to activated protein C was measured by determining the activated partial-thromboplastin times in the absence and presence of activated protein C (Coatest activated protein C resistance kit, Chromogenix, Molndal, Sweden). The results were expressed as the ratio of the two values. Plasma samples from 33 normal subjects were tested, and the mean (± 2 SD) ratio was found to be 2.7 ± 0.4. A ratio of less than 2.1 but not less than 1.6 was considered suggestive of heterozygosity for factor V Leiden, and a ratio of less than 1.6 was considered consistent with homozygosity.

Detection of the R506Q Mutation of the Factor V Gene

Blood samples were collected in EDTA, and genomic DNA was prepared by standard techniques. A 206-base-pair (bp) DNA fragment of the factor V gene that includes nucleotide 1691 was amplified by the polymerase chain reaction (PCR) with the forward primer 5′CATACTACAGTGACGTGGAC3′ and the reverse primer 5′TGTTCTCTTGAAGGAAATGC3′. Digestion of this 206-bp fragment by *Mnl*I yielded three fragments (47, 36, and 123 bp) in the normal allele, and two fragments (47 and 159 bp) in the mutant allele. The PCR was carried out in 25 μl of reaction mixture consisting of buffer (10 mM TRIS–hydrochloric acid [pH 9.0], 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.1 percent Triton X-100, and 0.2 mg of bovine serum albumin per milliliter), 0.2 nM of each nucleoside triphosphate, 250 nM of each primer, 100 to 200 ng of the DNA sample, and 0.125 μl of *Taq* polymerase (Appligene, Illkirch, France). The PCR was performed in 30 cycles consisting of 30 seconds at 94°C, 120 seconds at 63°C, and 180 seconds at 72°C. Eight microliters of the 206-bp amplified DNA product was digested with 1 μU of *Mnl*I (New England Biolabs, Beverly, Mass.) at 37°C for three hours. Then the sample was subjected to electrophoresis on 4 percent NuSieve agarose gels (FMC, Rockland, Me.), and the fragments were visualized with ethidium bromide.

RESULTS

The pedigrees of the seven highly consanguineous families with homocystinuria are shown in Figure 1, which indicates the observed or inferred genotypes for homocystinuria and factor V Leiden, as well as other genetic disorders.

Case Histories

Family 1

Subject VI-1 from Family 1 presented at the age of three weeks with severe hypotonia and a bulging fontanelle. The presence of arterial and venous thrombosis and hydrocephalus was later determined on a computed tomographic (CT) scan of the brain and was confirmed at autopsy five months thereafter. Homocystinuria due to a methylenetetrahydrofolate reductase deficiency was diagnosed. The patient's activated protein C resistance ratio was 1.6. The parents, who were inferred to be obligate heterozygotes for homocystinuria, were also heterozygous for factor V Leiden.

Family 2

Subject VI-7 from Family 2 was homozygous for homocystinuria because of cystathionine β-synthase deficiency. At the age of eight years, after an eye operation for dislocated optic lenses, he had deep-vein thrombosis with massive pulmonary embolism. Heparin treatment was given, followed by warfarin for six months. The patient was found to be homozygous for factor V Leiden.

Family 3

Subject V-2 from Family 3 presented at the age of four weeks with severe hypotonia and failure to thrive. CT of the brain disclosed arterial and venous thrombosis and hydrocephalus, because of which a ventriculoperitoneal shunt was inserted. This infant was homozygous for methylenetetrahydrofolate reductase deficiency and had severe psychomotor retardation, and she died at the age of two years from aspiration pneumonitis. Her father (Subject IV-1) was found to be homozygous for factor V Leiden, and her mother (Subject IV-2) to be heterozygous.

Family 4

Subject VI-2 from Family 4 presented at the age of five years with dislocation of the optic lenses and was found to be homozygous for homocystinuria because of cystathionine β-synthase deficiency. At the age of 10, a markedly reduced plasma cobalamin level was also found. A defect of cobalamin absorption not corrected by the provision of the normal human intrinsic factor was suggestive of the Imerslund–Graesback syndrome.[25] His elevated plasma homocysteine levels were not substantially altered by monthly injections of cobalamin. This child, who had both homocystinuria and selective vitamin B_{12} malabsorption, was not found to have the factor V Leiden mutation and remained free of any evidence of thrombosis at the age of 14. He is mildly retarded.

Family 5

Subjects IV-5 and IV-6 from Family 5 both presented with bilateral dislocation of the optic lenses and developmental delay at the age of five years. They were found
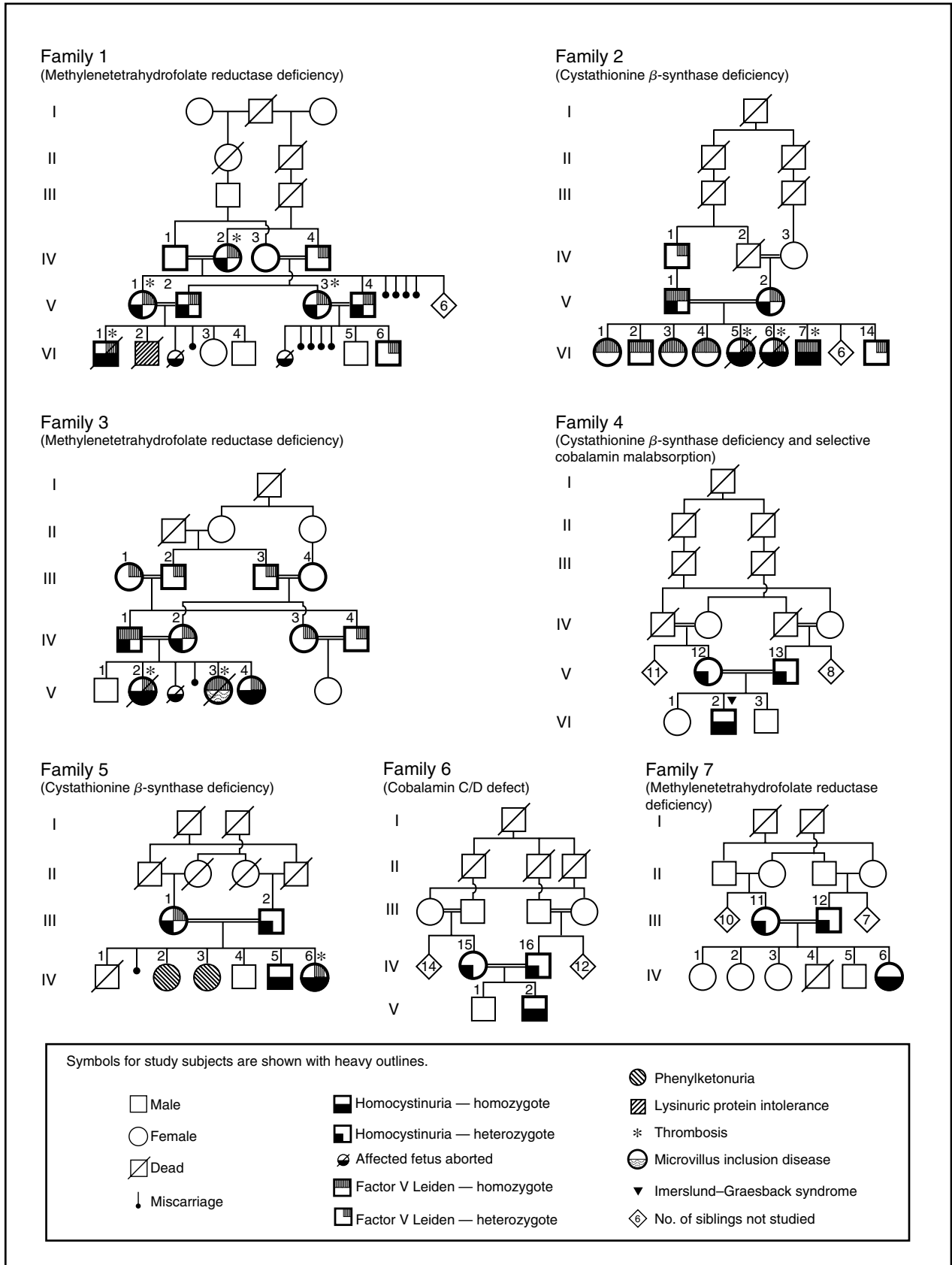
Figure 1. Pedigrees of Seven Families with Homocystinuria in Which the Factor V Leiden Mutation Was Studied.

to be homozygous for homocystinuria because of cysta-thionine β-synthase deficiency. At the age of 17, Subject IV-5 was found not to have factor V Leiden and has not had any thrombosis. In contrast, Subject IV-6, who was heterozygous for factor V Leiden, presented at the age of six years with left hemiparesis and in the ensuing years had three episodes of deep-vein thrombosis. At the age of 15, she was blind, severely retarded, and bed-ridden.

Family 6

Subject V-2 from Family 6 had generalized hypoto-nia and vomiting, beginning in the first week of life. At the age of three weeks, he was admitted to the hospital with hypothermia and seizures. Laboratory studies re-vealed pancytopenia, macrocytosis (mean corpuscular volume, 105 μm^3), and elevated plasma homocysteine and methylmalonic acid levels. The diagnosis of a co-balamin C/D defect was confirmed by study of his fibro-blasts. A CT scan of the brain showed no abnormality and no evidence of vascular occlusion. This child did not carry the factor V Leiden mutation and at the age of three years had not had thrombosis. He has severe mental retardation.

Family 7

Subject IV-6 from Family 7 presented at the age of three months with a bulging fontanelle and severe hypo-tonia. A CT scan of the brain revealed hydrocephalus but no evidence of cerebral thrombosis. A ventriculo-peritoneal shunt was inserted. The diagnosis of homozy-gosity for homocystinuria due to a methylenetetrahydro-folate reductase deficiency was confirmed by study of her fibroblasts. At the age of one year, the patient had only moderate developmental retardation. This infant does not carry the factor V Leiden mutation.

Association of Thrombosis, Homocystinuria, and Factor V Leiden

Table 1 lists the clinical and laboratory features of the 11 patients homozygous for homocystinuria from these seven families. Thromboembolic manifestations were ob-served in six patients, of whom four had cystathionine β-synthase deficiency and presented with thrombosis at the ages of seven or eight years. The remaining two had methylenetetrahydrofolate reductase deficiencies and thrombosis in early infancy. The thrombotic manifesta-tions were mainly venous. However, arterial thrombosis was also documented by brain CT in two patients (and in one of these also by autopsy) and was suggested in two others by the clinical presentation of acute hemi-paresis. In four of the six patients with homocystinuria who had thromboses, the events occurred in associa-tion with diarrhea, dehydration, or surgery.

All six patients with homocystinuria who had throm-boses were homozygous or heterozygous for factor V Leiden: one (Subject VI-7 in Family 2) was a homozy-gote; three (Subjects VI-5 and VI-6 in Family 2 and Subject V-2 in Family 3) were offspring of fathers ho-mozygous for factor V Leiden and heterozygous moth-ers and thus could be either homozygotes or heterozy-gotes; and two patients (Subject IV-6 in Family 5 and Subject VI-1 in Family 1) were determined to be hetero-zygotes. In an additional patient (Subject V-4 in Family

Table 1. Clinical and Laboratory Data on 11 Patients with Homozygous Homocystinuria.*

Family No./ Subject No.	Sex	Enzymatic Defect	Plasma Homocysteine (μmol/liter)	APC Resistance Ratio	Factor V Leiden Mutation	Thrombosis			Clinical Outcome
						Site	Age at Onset (yr)	Possible Trigger	
1/VI-1	M	MTHFR	50	1.6	ND†	Brain	0.2	Diarrhea	Death at 18 mo
2/VI-5	F	CBS	NPS	ND	Homozygous or heterozygous	Deep vein, lung	7	Diarrhea	Death at 8 yr
2/VI-6	F	CBS	NPS	ND	Homozygous or heterozygous	Deep vein, lung, brain	8	—	Death at 13 yr
2/VI-7	M	CBS	290	1.3	Homozygous	Deep vein, lung	8	Surgery	Mild mental retardation at 14 yr
3/V-2	F	MTHFR	47	ND	Homozygous or heterozygous	Brain	0.2	—	Death at 2 yr
3/V-4	F	MTHFR	62	1.6	Heterozygous	—	—	—	Healthy at 18 mo with warfarin
4/VI-2	F	CBS	350	2.2	None	—	—	—	Mild mental retardation at 14 yr
5/IV-5	M	CBS	322	3.4	None	—	—	—	Moderate mental retardation at 17 yr
5/IV-6	F	CBS	274	2.0	Heterozygous	Deep vein, brain	7	Surgery	Severe mental retardation at 15 yr
6/V-2	M	Cobalamin C/D	43	2.4	None	—	—	—	Severe mental retardation at 3 yr
7/IV-6	F	MTHFR	79	2.1	None	—	—	—	Moderate developmental retardation at 1 yr

*APC denotes activated protein C, MTHFR methylenetetrahydrofolate reductase, CBS cystathionine β-synthase, NPS a positive urinary nitroprusside test (indicating elevated levels of homocysteine), and ND not determined. In patients described as homozygous or heterozygous for the factor V Leiden mutation, the genotype was deter-mined by inference from the parents' factor V genetic status.

†This patient was determined to be heterozygous for the mutation on the basis of the plasma APC ratio.

3), homocystinuria was diagnosed prenatally and heterozygosity for factor V Leiden was identified postnatally. This infant was treated with warfarin, betaine, and leucovorin beginning at birth, and was healthy at 18 months.

Of the remaining four patients with homocystinuria (who were 1 to 17 years old at this writing), none has had thrombosis to date, even though two of them have had extremely high plasma homocysteine levels. None was found to carry the factor V Leiden mutation.

Among the 45 people we studied in the seven families, 33 had at least one of the mutant genes — either factor V Leiden or one of the defects causing homocystinuria. As Table 2 shows, thrombosis occurred only in family members who had a combination of defects, with the probable exception of one patient (Subject V-3 in Family 3) who had familial microvillus inclusion disease[26]; in this patient, recurrent thrombosis of the large veins was induced by central venous catheterization. She did not have homocystinuria but was homozygous for factor V Leiden. Her methylenetetrahydrofolate reductase genotype was not determined.

Interestingly, three women, all in Family 1, were heterozygous for both methylenetetrahydrofolate reductase deficiency and factor V Leiden and had recurrent abortions with placental infarctions. Of 25 pregnancies in these women, 9 ended in spontaneous abortion and 7 were associated with placental infarctions and led to the birth of newborns small for their gestational ages.

The values for protein C, protein S, and antithrombin III were in the normal range in 43 persons we studied. The two subjects not tested were the patients homozygous for homocystinuria in Family 2 (Subjects VI-5 and VI-6) who could be inferred to be either homozygous or heterozygous for factor V Leiden on the basis of their parents' genotypes.

DISCUSSION

Thromboembolism, venous, arterial, or both, is a major cause of death in patients homozygous for homocystinuria.[2] However, among 629 such patients described in one survey, only 158 presented with thromboembolic events.[2] Our data provide evidence that one reason for the variability of thrombosis in patients with homocystinuria is the presence or absence of factor V Leiden. A remarkable finding in the families we studied was that major thrombotic events occurred only in patients homozygous for homocystinuria who were also homozygous or heterozygous for factor V Leiden.

The likelihood that additional families with these two genetic defects will be identified is not remote, in view of the relatively high allelic frequency of factor V Leiden.[8,12] The high prevalence of the mutation in the families we have described is probably due to their high rates of consanguinity. The family pedigrees (Fig. 1) show a clustering of various other genetic diseases, reflecting the long history of intermarriage in this population.

Several recent reports have indicated that the concurrence of factor V Leiden and other genetic defects is frequent among patients with thrombophilia. Among

Table 2. Association between Mutated Genotypes for Homocystinuria and Factor V Leiden and the Occurrence of Thrombosis in 33 Subjects from the Seven Families Studied.

GENETIC ASSOCIATION		NO. OF SUBJECTS	THROMBOSIS	NO THROMBOSIS
HOMOCYSTINURIA	FACTOR V LEIDEN			
Homozygote	Homozygote	1	1	
Homozygote	Homozygote or heterozygote	3	3	
Homozygote	Heterozygote	3	2	1*
Homozygote	Normal genotype	4		4
Obligate heterozygote	Heterozygote	8	3†	5
Obligate heterozygote	Normal genotype	7		7
Obligate heterozygote	Homozygote	2		2
Not determined	Homozygote	5	1‡	4
All subjects		33	10	23

*This subject (Subject V-4 in Family 3) was an 18-month-old child treated with warfarin from birth.

†These three subjects were women who had recurrent abortions, low-birth-weight babies, and placental infarctions. One woman (Subject IV-2 in Family 1) was a grandmother who was considered to be heterozygous for homocystinuria on the basis of a high fasting plasma homocysteine level.

‡This subject (Subject V-3 in Family 3) had congenital diarrhea, and thrombosis was induced by central venous catheterization.

patients with deficient protein C, protein S, or antithrombin III activity, 15 to 26 percent also had the factor V Leiden mutation.[14,15,27-29] These double defects conferred a higher risk of thrombosis than did either defect alone, and the initial presentation of thrombosis occurred at an earlier age.[14,27,28]

Exogenous factors, such as surgery, diarrhea, trauma, immobilization, and pregnancy, can trigger thrombosis in patients who are heterozygous for protein C, protein S, or antithrombin III deficiency,[30] as well as in patients with factor V Leiden.[8,31,32] We found that such environmental risk factors also increased the tendency to thrombosis among patients homozygous for homocystinuria who have factor V Leiden.

Among women who are obligate heterozygotes for homocystinuria, the incidence of fetal loss may be increased,[2] and a recent report suggests that there may also be an increased perinatal mortality rate in their offspring.[33] The results of tests for resistance to activated protein C in the women in these studies were not reported. In our series, three women who were heterozygous for both homocystinuria and factor V Leiden had recurrent miscarriages, low-birth-weight babies, and placental infarctions. None had elevated plasma levels of antiphospholipid antibodies.[34]

High concentrations of homocysteine can induce the activation of factor V in endothelial cells[35] and inhibit the activation of protein C,[36,37] compromising a major mechanism by which blood coagulation is controlled. The increased tendency to thrombosis in patients with the combination of homocystinuria and factor V Leiden could result from an additive adverse effect of these two defects on a common protective mechanism in the coagulation cascade.

Mild-to-moderate increases in plasma levels of ho-

mocysteine have been thought to be an independent risk factor for arterial vascular disease[38-40] and recurrent venous thrombosis.[41,42] However, none of the 16 obligate carriers of cystathionine β-synthase deficiency or methylenetetrahydrofolate reductase deficiency whom we studied have presented with arterial disease or venous thrombosis to date. Most of these subjects were less than 50 years old when studied, and two of them were also homozygous and two heterozygous for factor V Leiden. These observations suggest that additional contributing factors may be needed for thrombosis to occur.

Our observations, as well as those of others, imply that a search for other hereditary thrombotic disorders should be conducted in patients who are found to carry mutant genes predisposing them to thrombosis. Patients with more than one mutation should be evaluated carefully before they undergo surgical, medical, or obstetrical procedures that carry an increased thrombotic risk, since they may require regimens of appropriate prophylactic anticoagulant therapy.

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