Linkage analysis demonstrates that the prothrombin G20210A mutation jointly influences plasma prothrombin levels and risk of thrombosis

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Association studies suggest that the G20210A mutation (G to A substitution at nucleotide position 20210) in the prothrombin gene (*PT*) is associated with increased plasma prothrombin activity and with increased risk for venous thromboembolism. To test directly for linkage between this *PT* variant and plasma prothrombin activity we performed a family-based study. The G20210A genotypes and plasma prothrombin activity levels were determined in 435 individuals belonging to 22 extended Spanish families. The sample was composed of 388 homozygous (*G/G*) normal individuals and 43 heterozygote (*G/A*) and 4 homozygote (*A/A*) carriers for the G20210A mutation. The results of variance-component linkage analysis yielded a highly significant lod score of 3.6 ($P = 2.4 \times 10^{-5}$) between this mutation and a quantitative trait locus (QTL) that influences prothrombin activity. Importantly, a conditional linkage analysis that simultaneously accounted for association with the G20210A variant completely eliminated the linkage signal, which indicates that this mutation affects the function of the prothrombin gene. Additionally, a bivariate linkage analysis of plasma prothrombin activity and thrombosis significantly improved the linkage signal for prothrombin activity (lod score = 4.7; $P = 1.5 \times 10^{-6}$) and provided strong evidence that this QTL has a pleiotropic effect on the risk of thrombosis (lod score = 2.43; $P = .0004$). These results represent the first direct genetic evidence that a QTL in the *PT* gene influences prothrombin activity levels and susceptibility to thrombosis and strongly support the conclusion that G20210A is a functional polymorphism. (Blood. 2000;95:2780-2785)

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Introduction

Thrombosis is a disease of significant public health importance. It is clear that genetic and environmental factors play a critical role in its etiology.[1] Recently there has been a growing interest in finding genetic polymorphisms that are associated with an increased risk of thrombosis, and a number of polymorphisms in candidate genes have been implicated.[2-5] One such polymorphism is a genetic variation in the 3′-untranslated region of the prothrombin structural (*PT*) gene involving a G to A substitution at nucleotide position 20210 (G20210A). This genetic variant is associated with an increased risk of thrombosis.[2] Compared with normal homozygotes (*G/G*), heterozygous (*G/A*) carriers of the mutation have an almost 3-fold increased risk of venous thrombosis.[2,6-11]

Another important finding associated with this *PT* polymorphism is the significant variability of prothrombin plasma levels as a function of G20210A genotypes. For example, the mutant *A/A* homozygotes exhibit the highest values.[12] Using data from Poort et al,[2] we can calculate that at least 6.7% of the phenotypic variance in prothrombin levels is attributable to this chromosomal region in the Dutch population. In a family-based study, we previously demonstrated that prothrombin levels have a significant genetic component, with a heritability of 49%.[13] This high heritability indicates that genetic factors play an important role in determining prothrombin levels in the general population and also suggests that other polymorphisms in addition to *PT* G20210A are likely to have an influence on prothrombin levels.

Unfortunately, the pathogenic mechanism associated with the G20210A mutation is unknown. The observed sequence change, which substitutes a GA for a CA at or near the cleavage and polyadenylation site, may induce a relatively higher translation efficiency or higher stability of the transcribed messenger RNA (mRNA).[2] Alternatively, the 20210 *A* allele may not be functionally distinct. The observed association may represent an indirect correlation that is due to linkage disequilibrium of the G20210A site, with another sequence variation inside the *PT* gene that is responsible for elevated prothrombin levels. However, 2 studies[2,14] have failed to identify sequence polymorphisms in either the promoter (from −1050 base pair [bp]) or coding region of the *PT* gene that are in linkage disequilibrium with the G20210A mutation.

To date, thrombosis-related genetic epidemiological research has concentrated on classical association studies in which polymorphic variations of candidate genes were assessed in unrelated individuals.[2-5] Typically, case-control comparisons were performed to evaluate whether the candidate locus had any effect on the risk of disease. Such studies suffer from a major epidemiological weakness because estimates of a genetic effect are usually too low and markedly underestimate the relative importance of the candidate

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gene. Also, from such studies it is impossible to determine unequivocally if the effects are due to linkage disequilibrium[15,16] or if the marker is itself a susceptibility factor. These studies are also prone to type I errors, or a false or mistaken result, due to hidden population genetic heterogeneity. In contrast, the power of family-based genetic analyses comes from the possibility to localize directly and evaluate accurately the potential effect of a disease locus through linkage analysis. Family-based studies can also exploit information on linkage disequilibrium to unequivocally determine whether a particular polymorphism is responsible for an observed linkage signal.[16] However, few studies[17-20] have used a family-based approach to demonstrate or exclude linkage between a putative disease locus influencing risk of thrombosis and candidate genes (eg, factor V Leiden mutation or *PROS* gene).

Given the paucity of information in this respect concerning the *PT* G20210A polymorphism, we performed a family-based linkage/disequilibrium analysis using extended Spanish kindreds. Because the Spanish population exhibits one of the highest carrier frequencies (6.5%) of the G20210A mutation in the world,[6-8,10,11,21-24] it is an excellent population in which to conduct such a linkage analysis. Our main goals were to obtain unequivocal evidence of linkage, rather than mere association, of the *PT* polymorphism with a quantitative trait locus (QTL) that influences prothrombin levels and to determine whether this polymorphism is itself responsible for any genetic effects attributable to the *PT* gene.

Patients and methods

Enrollment of family members

The Spanish families available for our studies were provided by the GAIT (Genetic Analysis of Idiopathic Thrombophilia) Project. The GAIT Project has been described in detail elsewhere.[13] Briefly, our sample included 435 individuals: 397 individuals were from 21 families of the GAIT project,[13] and 38 individuals were from an additional family with identical criteria, which we recruited and used expressly for this study. The depth and complexity of these pedigrees is illustrated in Table 1. To be included in the study, a family had to have at least 10 living individuals in 3 or more generations (ie, extended pedigrees). Thirteen families were selected through a proband with idiopathic thrombophilia, which was defined as either multiple thrombotic events (at least 1 of which was spontaneous), a single spontaneous episode of thrombosis with a first-degree relative also affected, or onset of thrombosis before 45 years of age. The proband's thrombophilia was considered idiopathic because all known (during the recruitment period of 1995-1996) biological causes of thrombophilia were excluded including antithrombin deficiency, protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, heparin cofactor II deficiency, factor V Leiden, dysfibrogenemia, lupus anticoagulant, and antiphospholipid antibodies. These thrombophilic factors were also absent in all affected relatives of the probands. We randomly selected 9 families as controls without regard to phenotype, but with similar pedigree size as that of the pedigrees ascertained through thrombophilic probands.

The subjects were interviewed by a physician to determine their health and reproductive history, current medications, and use of sex hormones (oral contraceptives or hormonal replacement therapy) and whether they were current or former smokers. The subjects were also questioned about previous episodes of venous or arterial thrombosis, the age at which these events occurred, and the presence of potentially correlated disorders such as diabetes and lipid disease. All of the procedures were reviewed by the Institutional Review Board of the Hospital de Sant Pau, Barcelona, Spain. Adult subjects gave informed consent for themselves and for their minor children.

Table 1. Examined relative pairs in the 22 pedigrees

n	Relationship	Degree of Relationship
435	Self	0
1	Monozygotic twin pair	0
497	Parent-offspring	1
361	Siblings	1
236	Grandparent-grandchild	2
770	Avuncular	2
4	Half-siblings	2
13	Great grandparent-grandchild	3
162	Grand avuncular	3
669	First cousins	3
9	Great grand avuncular	4
325	First cousins, once removed	4
79	Second cousins	5

Blood collection and phenotype analyses

Blood was obtained from the antecubital vein following a 12-hour fast. Samples for hemostatic tests were collected in 1/10 volume of 0.129 mol/L sodium citrate. None of the individuals were being treated with anticoagulant therapy at the time of sample collection. Assays for activated partial thromboplastin time (APTT), prothrombin time (PT), and coagulation factors were performed immediately on fresh plasma samples. The remaining plasma samples were stored at −80°C until use.

The following phenotypes were measured as previously described [13]: APTT; PT; fibrinogen; coagulation factors including prothrombin, which was assayed using deficient plasma (Diagnostica Stago, Asnières, Spain); functional, free, and total protein S; activated protein C resistance; antithrombin; protein C; heparin cofactor II; plasminogen; plasminogen activator inhibitor; tissue plasminogen activator; dimerized plasmin fragment D (D-dimer); tissue factor; von Willebrand factor; levels of histidine-rich glycoprotein; and tissue factor pathway inhibitor. To reduce measurement error, all assays were performed in duplicate, and the average value was calculated for each person. Intra-assay and inter-assay coefficients of variation were generally estimated between 2% and 6%.

Genotype analysis

DNA was extracted using the standard protocols.[25] We genotyped the *PT* G20210A and factor V Leiden mutations in 435 individuals using the 4 primers previously described[26] in a multiplex–polymerase chain reaction (multiplex-PCR), with minor modification in the reaction conditions. Briefly, we used a 50-μL mixture containing 20 mmol/L Tris-HCl (tris[hydroxymethyl] aminomethane–hydrochloride, pH 8.2), 2 mmol/L magnesium chloride (MgCl), 0.2 mmol/L of each nucleoside 5′-triphosphate, 0.5 μmol/L of each primer, 250 ng DNA, and 1.5 units *Taq-gold* (Perkin Elmer, Norwalk, CT). The mixture was subjected to 40 cycles at 95°C for 10 minutes, 55°C for 1 minute, and 72°C for 1 minute, with a final extension step of 72°C for 10 minutes. The 175-bp and 118-bp PCR products were digested with *TaqI* (Biolabs, Beverly, MA) and electrophoresed on a 3% Nusieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The genotypic data were entered into a database and were analyzed for discrepancies (ie, violations of Mendelian inheritance), using the program INFER available in PEDSYS, the pedigree-oriented database system (Southwest Foundation for Biomedical Research, San Antonio, TX).[27]

Allelic frequency and identical-by-descent (IBD) probability estimation

Because the sample contained related individuals, we used a maximum likelihood method, which accounted for pedigree structure, and a computer program package (SOLAR, Southwest Foundation for Biomedical Research, San Antonio, TX)[28] to estimate allelic frequencies at the *PT* locus. After obtaining these maximum likelihood estimates, we estimated the probabilities of sharing alleles that are identical-by-descent (IBD) for all pairs of individuals using the likelihood-based approach described in Almasy and Blangero.[28] The resulting matrix of IBD probabilities was then used in all subsequent linkage analyses.

Linkage analysis

Pedigree-based variance component linkage analyses were performed (*SOLAR*).[28] The variance component method uses the correlation in phenotype between relatives to partition the trait variance into components attributable to the additive effects of unspecified genes; the effects of genes in the region of linkage; and a residual component consisting of environmental effects, measurement error, and nonadditive genetic effects such as dominance. Information on genome-wide additive genetic effects (ie, heritability) comes from the kinship between family members, while linkage information regarding specific quantitative trait loci is provided by estimates of IBD allele sharing between individuals for each genetic marker tested. Sex, sex-specific age, and age-squared were included as covariates in all of the analyses. Variance component-based linkage analysis of the discrete trait, thrombosis affection status, was performed similarly using a threshold model as described elsewhere.[29] A bivariate linkage analysis of plasma prothrombin activity and thrombosis, which uses the correlations between phenotypes to test hypotheses of pleiotropy and to improve power to detect linkage, was performed using the mixed discrete/continuous trait multivariate model of Williams et al.[30]

Combined linkage/disequilibrium analysis

Quantitative trait association analysis (*SOLAR*)[28] was performed using the measured genotype approach[31] by testing for genotype-specific differences in the means of traits while allowing for nonindependence among family members. To assess linkage and association simultaneously,[16,32] an extension of the variance component-based linkage test was performed by simultaneously incorporating the genotype-specific means of the measured genotype test. These analyses were performed using the SOLAR package.[28] If a variant is functional and there are no other functional variants in the candidate gene under investigation, then a linkage analysis that is performed conditional on the measured genotypes (ie, a linkage test in which the measured genotypes are controlled for) should yield no evidence for linkage. This is because all genetic variance that is due to the QTL will be removed when the QTL is itself used as a covariate. Alternatively, if a variant is merely in linkage disequilibrium with a functional site, linkage analyses will have additional predictive power over the measured genotype test and will yield evidence for linkage.

Hypothesis testing and parameter estimation

Variance component parameters were estimated through maximum likelihood methods, and hypothesis testing was performed using likelihood ratio test statistics.[33,34] As some families were ascertained through thrombophilic probands, all analyses were performed with an ascertainment correction to allow unbiased estimation of parameters relevant to the general population. This was achieved by conditioning the likelihood of the pedigree on the phenotype of the proband.[31,35]

Results

The composition of the families studied, including their ascertainment, sex, the number of individuals, and whether or not an individual was affected with thrombosis, and the G20210A genotypes are given in Table 2. Among the 435 individuals included in our sample, 57 individuals had venous or arterial thrombosis. Of these, 51 were members of the families ascertained through thrombophilic probands, and 6 were from the randomly ascertained families. The age at diagnosis of first thrombosis ranged from 12-76 years, with a mean of 44.5 years. Of the 57 affected people, 16 (28%) had multiple thrombotic events.

The individuals were genotyped for the G20210A allelic variant by PCR. We found 388 homozygotes for the common variant (*G/G*), 43 heterozygotes (*G/A*), and 4 homozygotes for the *A* allele (*A/A*). The rare *A/A* homozygotes were members of families 5 and 22 (Table 2) and represented 1 parent-offspring pair and 1 sibling pair, respectively. Likelihood-based analysis of the genotype data, which allows for the nonindependence of genotypes among relatives, yielded a maximum-likelihood estimate of the 20210A allele frequency equal to 0.0304 in these families. Assuming the Hardy-Weinberg equilibrium, this allele frequency predicts a heterozygote frequency of 0.0590, which is consistent with previous estimates for the Spanish population.[6,36]

Table 2. Composition of 22 families who participated in the study

Family Number	Ascertained	Number of Individuals	Male/ Female	Affected Thrombosis	G/G	G/A	A/A
1	Thrombosis	21	15/6	4	21	0	0
2	Thrombosis	38	19/19	4	38	0	0
3	Thrombosis	13	5/8	3	13	0	0
4	Thrombosis	11	6/5	4	11	0	0
5	Thrombosis	10	6/4	2	2	6	2
6	Thrombosis	33	10/23	7	32	1	0
7	Thrombosis	20	10/10	4	14	6	0
8	Thrombosis	14	5/9	5	14	0	0
9	Thrombosis	13	8/5	4	13	0	0
10	Thrombosis	14	7/7	3	14	0	0
11	Control	22	15/7	2	15	7	0
12	Control	27	14/13	0	26	1	0
13	Control	22	10/12	0	22	0	0
14	Control	15	6/9	1	15	0	0
15	Control	14	3/11	0	14	0	0
16	Control	24	10/14	0	24	0	0
17	Control	17	11/6	0	13	4	0
18	Control	22	7/15	3	22	0	0
19	Control	20	9/11	0	20	0	0
20	Thrombosis	18	7/11	3	18	0	0
21	Thrombosis	9	3/6	4	9	0	0
22	Thrombosis	38	20/18	4	18	18	2
Total		435	207/228	57	388	43	4

The estimated heritability of prothrombin activity level was 0.51 ± 0.09. This is consistent with our previous estimate of 0.49 ± 0.088 in a subset of these families.[13] Linkage analysis using the maximum-likelihood estimate of the IBD probability matrix for the G20210A variant yielded a lod score of 3.6 ($P = 2.4 \times 10^{-5}$) between the G20210A variant and a putative QTL that influences the prothrombin activity levels at a recombination fraction of 0.0. The results support the presence of a major QTL in the region of the *PT* gene. Given this unequivocal evidence for linkage, we performed an association analysis to test for linkage disequilibrium between the QTL and the G20210A variant. This measured genotype analysis revealed significant differences among the 3 genotypes in their plasma prothrombin values: $GG = 123.6 \pm 1.8$, $GA = 141.1 \pm 3.7$, and $AA = 167.7 \pm 8.4$; $P < 1 \times 10^{-7}$. Figure 1 shows the prothrombin activity levels related to the 3 genotypes.

When we performed a combined linkage/disequilibrium analysis that accounted for the association of QTL with the mutation, there was no evidence (lod = 0.0) of additional functional polymorphisms in the *PT* gene. Under the assumption that the G20210A mutation affects the function of the *PT* locus, we calculated that this mutation accounts for 6.1% of the variance in prothrombin activity levels in this population. Although the G20210A mutation has a highly significant effect on prothrombin activity, there remains substantial residual genetic variation in prothrombin activity even after taking into account the G20210A mutation. This indicates that there are other genes that influence prothrombin activity level.

Because high prothrombin levels appear to predispose to

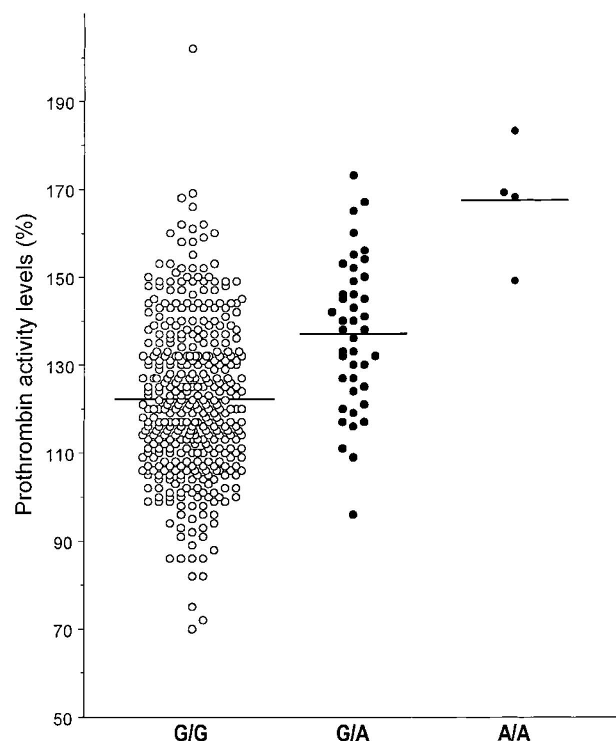

Figure 1. The percent of prothrombin antigen levels according to the 3 genotypes for the G20210A mutation. The horizontal bar indicates the mean level in each group.

thrombosis,[2] we thought that it would be prudent to examine the possible effect of this QTL on the risk of thrombosis. Among the 43 heterozygous (*A/G*) individuals, 9 individuals were symptomatic, and 34 were free of thrombosis at the time of examination. Among the 4 *A/A* individuals, only 1 had suffered from thrombotic disease. Therefore, among the 57 individuals with a history of thrombosis, the G20210A mutation was present in 10 individuals, ie, 1 homozygous (*A/A*) and 9 heterozygous (*A/G*) individuals. The 47 symptomatic members of the remaining families were *G/G* homozygotes, and their genetic risk factors for thrombosis have not been determined. Because of the variation in age-at-onset of thrombosis and the nonindependence of the related individuals in the sample, simple tabulations comparing genotype frequencies were not appropriate. To obviate this problem, we performed analyses of thrombosis using a normal threshold model that allows for sex- and age-specific risks. The threshold model is very similar to a standard logistic regression model that is commonly used in epidemiological studies. However, threshold models are superior to standard logistic regression models for family-based studies because they allow for correlation among family members.

We performed a variance-component linkage analysis for the presence of thrombosis with the G20210A mutation using the normal threshold model. The heritability of thrombosis risk was estimated as 0.64 ± 0.13. The lod score for this marginal analysis was only suggestive of linkage (lod = 0.44; $P = .077$). Association analysis using the measured genotype approach provided evidence for significant differences in risk among G20210A genotypes ($P = .03$). The results from the measured genotype analysis can be translated into standardized odds ratios. For example, the expected odds ratio for 50-year-old heterozygote male carriers is 3.27. Thus, a heterozygote would be approximately 3 times as likely to experience a thrombotic event than would an individual homozygous for the *G* allele. This estimate is consistent with earlier estimates of the risk associated with this mutation.[2]

Given the potential correlation between prothrombin levels and the risk of thrombosis, we performed a joint bivariate linkage analysis using both of these phenotypes simultaneously. This bivariate linkage analysis of plasma prothrombin activity and thrombosis significantly strengthened the linkage signal for prothrombin activity (lod = 4.7; $P = 1.5 \times 10^{-6}$) and provided strong evidence that this QTL has a pleiotropic effect on the risk of thrombosis (lod = 2.43; $P = .0004$). This improvement in linkage signal is a direct function of the correlation between these 2 phenotypes, which exhibited a genetic correlation of 0.24 in this sample.

Discussion

This is the first large family-based study to measure the effect of the G20210A mutation. Our approach was to gather and analyze data using extended pedigrees that were systematically collected to allow for ascertainment correction and general population inferences. Most of the knowledge regarding the genetic factors involved in common thrombosis has come from association studies that employed case-control designs to look at known polymorphic variations in candidate genes.[1-3,37] Although such studies provide important evidence for genetic effects, they are limited to known candidate genes, and they are susceptible to type I errors due to hidden population stratification. In addition, they do not have the advantage of exploiting genetic transmission in pedigreed families, and thus they are unable to reliably estimate the relative importance of genetic factors in determining within-population variation in thrombosis risk.[38] Family-based studies eliminate these problems, although their costs tend to be greater.

Our study confirms and extends the previously indirect observation that individuals with different genotypes for the G20210A mutation showed significant differences in their plasma prothrombin levels. The additive genetic heritability of plasma prothrombin levels is relatively high (greater than 50%), indicating that a substantial portion of the phenotypic variation in plasma prothrombin levels is due to the additive effects of genes.

In this study we have extended our previous results by documenting the close linkage between a QTL influencing prothrombin levels and the prothrombin gene (specifically the G20210A mutation). The observed lod score of 3.6 is highly significant and provides strong evidence for the existence of a QTL influencing prothrombin activity. The complete elimination of evidence for linkage, when we simultaneously allowed for an association between the G20210A mutation and prothrombin levels, is consistent with a direct functional effect of this locus. The G20210A mutation accounts completely for the genetic linkage signal we observed. Although we cannot exclude the possibility that part of the remaining genetic variance is due to unknown mutations in the prothrombin gene, the most probable origin should be other unknown genes. This result supports our previous observation that multiple genes of varying effects will be involved in determining variation in hemostasis-related phenotypes.[13]

Our results support the hypothesis that G20210A is a functional mutation. They are consistent with previously reported studies that failed to find other polymorphisms in the coding region and in the −1050-bp region of the promoter of the prothrombin gene, which might be in linkage disequilibrium with the G20210A mutation.[2,14]

However, the mechanisms by which the G20210A mutation causes the increase in plasma prothrombin levels and by which it confers an increased risk of thrombosis remain to be elucidated. Both the location of the G20210A mutation in the 3′-UT region of the prothrombin gene and our result demonstrating that this mutation accounts for the majority of variability observed in prothrombin levels that can be assigned to this chromosomal region suggest that the mutation is involved in some regulatory mechanism that may be related to translation efficiency or higher stability of the transcribed mRNA. Further studies are necessary to address these questions.

We have demonstrated that this QTL also has a pleiotropic effect on the risk of thrombosis. In these analyses we have used a broad definition of thrombosis that includes both venous and arterial events. We recognize that this is a controversial strategy. However, there is a growing body of evidence to suggest that the genetic influences on venous and arterial thrombosis may overlap substantially. While the environmental triggers of venous and arterial events may differ, many of the underlying biological risk factors are the same. Levels of homocysteine,[39,40] factor VIII, and von Willebrand factor[41,42] are all correlated with the risk of both venous and arterial forms of thrombosis. Additionally, analyses from The GAIT Project suggest that the genetic correlation between venous and arterial thromboses is not significantly different from one that indicates that these 2 conditions share the majority of their genetic influences (unpublished data). In any case, if venous and arterial thromboses are genetically distinct, including them both in the same genetic analyses would be conservative, thereby reducing apparent genetic effects rather than magnifying them.

Our predicted risk ratio for thrombosis in G20210A heterozygous individuals is consistent with the previous estimate[2] and indicates at least a 3-fold increase in the risk of thrombosis. Our bivariate analysis was able to substantially enhance this linkage signal by simultaneously exploiting the quantitative variability of plasma prothrombin levels in tandem with the risk of thrombosis. This approach is based on the exploitation of the continuous relationship between such risk factors and disease status and has been shown to dramatically increase the power to detect and accurately localize QTLs.[30] The current results confirm the valuable potential of this approach as a basic tool for mapping the genes of complex diseases.

In summary, these results represent the first direct genetic evidence that a QTL in the *PT* gene influences prothrombin activity levels and susceptibility to thrombosis and strongly support the conclusion that G20210A represents a functional polymorphism.

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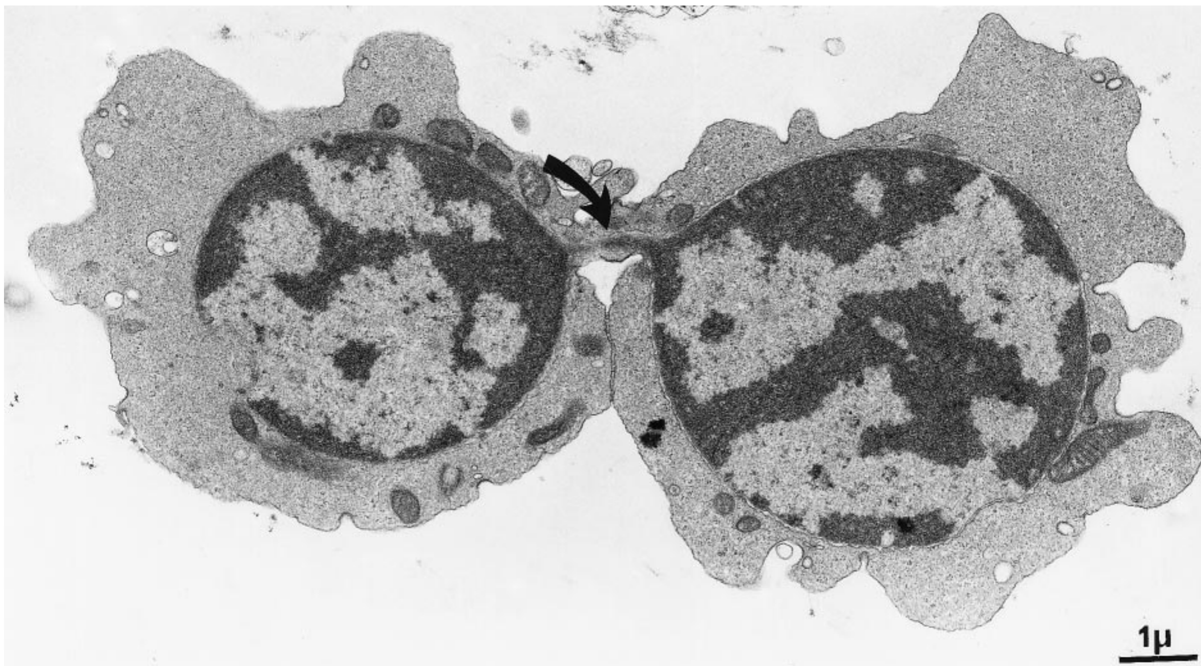
References

1. Rosendaal FR. Risk factors for venous thrombosis: prevalence, risk, and interaction. Semin Hematol. 1997;34:171.
2. Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3í-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase venous thrombosis. Blood. 1996;88:3698.
3. Gonzalez-Conejero R, Lozano ML, Rivera J, et al. Polymorphisms of platelet membrane glycoprotein Ibβ associated with arterial thrombotic disease. Blood. 1998;92:2771.
4. Rosendaal FR, Koster T, Vandenbroucke JP, Reitsma PH. High risk of thrombosis in patients homozygous for factor V Leiden (APC resistance). Blood. 1995;85:150.
5. Spek CA, Koster T, Rosendaal FR, Bertina RM, Reitsma PH. Genotypic variation in the promoter region of the protein C gene is associated with plasma protein C levels and thrombotic risk. Arterioscler Thromb Vasc Biol. 1995;15:214.
6. Souto JC, Coll I, Llobet D. The prothrombin 20210A allele is the most prevalent genetic risk factor for venous thromboembolism in the Spanish population. Thromb Haemost. 1998;80:366.
7. Brown K, Luddington R, Williamson D, Baker P, Baglin T. Risk of venous thromboembolism associated with a G to A transition at position 20210 in the 3′-untranslated region of the prothrombin gene. Br J Haematol. 1997;98:907.
8. Arruda VR, Annichino-Bizzacchi JM, Gonçalves MS, Costa FF. Prevalence of the prothrombin gene variant (nt20210A) in venous thrombosis and arterial disease. Thromb Haemost. 1997;78:1430.
9. De Stefano V, Chiusolo P, Paciaroni K, et al. Prevalence of the factor II G20210A mutation in symptomatic patients with inherited thrombophilia. Thromb Haemost. 1998;80:342.
10. Ferraresi P, Marchetti G, Legnani C, et al. The heterozygous 20210 G/A prothrombin genotype is associated with early venous thrombosis in inherited thrombophilias and is not increased in frequency in artery disease. Arterioscler Thromb Vasc Biol. 1997;17:2418-2422.
11. Hillarp A, Zöller B, Svensson PJ, Dahlbäck B. The 20210 A allele of the prothrombin gene is a common risk factor among Swedish outpatients with verified deep venous thrombosis. Thromb Haemost. 1997;78:990.
12. Souto JC, Mateo J, Soria JM, Coll I, Llobet D, Fontcuberta J. Homozygotes for prothrombin gene 20210 A allele in thrombophilic family without clinical manifestations of venous thromboembolism. Haematologica. 1999;84:627.
13. Souto JC, Almasy L, Borrell M, et al. Genetic determinants of hemostasia phenotypes in Spanish families. Circulation. In press.
14. Zivelin A, Rosenberg N, Faier S, et al. A single genetic origin for the common prothrombotic G20210A polymorphism in the prothrombin gene. Blood. 1998;92:1119.
15. Ruiz A, Barbadilla A. The contribution of quantitative trait loci and neutral marker loci to the genetic variances and covariances among quantitative traits in randomly mating populations. Genetics. 1995;139:445.
16. Almasy L, Williams JT, Dyer TD, Blangero J. QTL detection using combined linkage/disequilibrium analysis. Genet Epidemiol. 1999;17(suppl 1):531.
17. Bertina RM, Koeleman BPC, Koster T, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature. 1994;369:64.
18. Espinosa Y, Morell M, Souto JC, et al. Absence of linkage between type III protein S deficiency and the PROS1 and C4BP genes in families carrying the protein S Heerlen Allele. Blood. 1997;89:2799.
19. Hasstedt S, Bovill EG, Callas PW, Long GL. An unknown genetic defect increases venous thrombosis risk, through interaction with protein C deficiency. Am J Hum Genet. 1998;63:569.
20. Hennis BC, Van Boheemen PA, Koeleman BP, et al. A specific allele of the histidine-rich glycoprotein (HRG) locus is linked with elevated plasma levels of HRG in a Dutch family with thrombosis. Br J Haematol. 1995;89:845.
21. Rosendaal FR, Doggen CJM, Zivelin A, et al. Geographic distribution of the 20210 G to A prothrombin variant. Thromb Haemost. 1998;79:706.
22. Cumming AM, Keeney S, Bhavnani M, Shwe KH, Hay CRM. The prothrombin gene variant: prevalence in U.K. anticoagulant clinic population. Br J Haematol. 1997;98:353.
23. Howard TE, Marusa M, Boisza J, et al. The prothrombin gene 3′-untranslated region mutation is frequently associated with factor V Leiden in thrombophilic patients and shows ethnic-specific variation in allele frequency [letter]. Blood. 1998;91:1092.
24. De Maat MPM, Bladbjerg EM, Johansen LG, Gram J, Jespersen J. Absence of prothrombin mutation in Inuit (Greenland Eskimos). Thromb Haemost. 1998;79:882.
25. Miller SA, Dykes DD, Polesky HF. A simple salting

out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res. 1998; 6:1215.

26. Ripoll L, Paulin D, Thomas S, Drouet LO. Multiplex PCR-mediated site-directed mutagenesis for one-step determination of factor V Leiden and G20210A transition of the prothrombin gene. Thromb Haemost. 1997;78:960.
27. Dyke B. PEDSYS: a pedigree data management system [user's manual]. San Antonio, TX: Southwest Foundation for Biomedical Research; 1995. Population Laboratory, Department of Genetics, PGL Tech Rep, No. 2.
28. Almasy L, Blangero JC. Multipoint quantitative trait linkage analysis in general pedigrees. Am J Hum Genet. 1998;62:1198.
29. Duggirala R, Williams JT, Williams-Blangero S, Blangero J. A variance component approach to dichotomous trait linkage analysis using a threshold model. Genet Epidemiol. 1997;14:987.
30. Williams JT, VanEerdewegh P, Almasy L, Blangero J. Joint multipoint linkage analysis of multivariate qualitative and quantitative traits: I. Likelihood formulation and simulation results. Am J Hum Genet. 1999;65:1134.
31. Hopper JL, Matthews JD. Extensions to multivariate normal models for pedigree analysis. Ann Hum Genet. 1982;4:373.
32. Amos CI. Robust variance-components approach for assessing genetic linkage in pedigrees. Am J Hum Genet. 1994;54:535.
33. Kendall MG, Stuart A. The Advanced Theory of Statistics. Volume 2. Inference and Relationship. New York, NY: Hafner; 1967.
34. Self SG, Liang K-Y. Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. J Am Stat Assc. 1987;82:605.
35. Boehnke M, Lange K. Ascertainment and goodness of fit of variance component models for pedigree data. Prog Clin Biol Res. 1984;147:173.
36. Zabalegui N, Montes R, Orbe J, et al. Prevalence of FVR506Q and prothrombin 20210A mutations in the Navarrese population. Thromb Haemost. 1998;80:522.
37. Iacoviello L, Di Castelnuovo A, de Knijff P, et al. Polymorphisms in the coagulation factor VII gene and the risk of myocardial infarction. N Engl J Med. 1998;338:79.
38. Weiss KM. Genetic Variation and Human Disease. Cambridge: Cambridge University Press; 1993.
39. Nygard O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE. Plasma homocysteine levels and mortality in patients with coronary artery disease. N Engl J Med. 1997;337:230.
40. Den Heijer M, Koster T, Blom HJ, et al. Hyperhomocysteinemia as a risk factor for deep-vein thrombosis. N Engl J Med. 1996;334:759.
41. Koster T, Blann AD, Briët E, Vandenbroucke JP, Rosendaal FR. Role of clotting factor VIII in effect of von Willebrand factor on occurrence of deep-vein thrombosis. Lancet. 1995;345:152.
42. Folsom AR, Wu KK, Rosamond WD, Sharrett AR, Chambless LE. Prospective study of hemostatic factors and incidence of coronary heart disease. The atherosclerosis risk in communities (ARIC) study. Circulation. 1997;96:1102.



Crossing the bridge. This micrograph shows the thin chromatin bridge linking 2 erythroblasts in a case of congenital dyserythropoietic anemia type I. We used the opportunity to section this characteristic, but rare and discrete, phenomenon. Crossing the bridge was worth the snapshot. . . . Elisabeth M. Cramer and Josette Guichard, INSERM U.474, Hôpital de Port-Royal, Paris, France.