Genetic Determinants of Hemostasis Phenotypes in Spanish Families

Juan Carlos Souto, MD; Laura Almasy, PhD; Montserrat Borrell, PhD; Merce Garí, BSc; Elisabet Martínez, BSc; José Mateo, MD; William H. Stone, PhD; John Blangero, PhD; Jordi Fontcuberta, MD, PhD

Background—Recent studies have described genetic mutations that affect the risk of thrombosis as a result of abnormal levels of such hemostatic parameters as protein C, protein S, and the activated protein C resistance ratio. Although these mutations suggest that genes play a part in determining variability in some hemostasis-related phenotypes, the relative importance of genetic influences on these traits has not been evaluated.

Methods and Results—The relative contributions of genetic and environmental influences to a panel of hemostasis-related phenotypes were assessed in a sample of 397 individuals in 21 extended pedigrees. The effects of measured covariates (sex, age, smoking, and exogenous sex hormones), genes, and environmental variables shared by members of a household were quantified for 27 hemostasis-related measures. All of these phenotypes showed significant genetic contributions, with the majority of heritabilities ranging between 22% and 55% of the residual phenotypic variance after correction for covariate effects. Activated protein C resistance ratio, activated partial thromboplastin time, and Factor XII showed the strongest heritabilities, with 71.3%, 83.0%, and 67.3%, respectively, of the residual phenotypic variation attributable to genetic effects.

Conclusions—These results clearly demonstrate the importance of genetic factors in determining variation in hemostasis-related phenotypes that are components of the coagulation and fibrinolysis pathways and that have been implicated in risk for thrombosis. The presence of such strong genetic effects suggests that it will be possible to localize previously unknown genes that influence quantitative variation in these hemostasis-related phenotypes that may contribute to risk for thrombosis. (Circulation. 2000;101:1546-1551.)

Key Words: genetics ■ coagulation ■ fibrinolysis ■ epidemiology ■ thrombosis

The physiological and biochemical pathways involved in hemostasis are complex. However, recently, important advances have been made in characterizing the major phenotypic components of the coagulation and fibrinolysis pathways. Epidemiological studies have focused on correlations among hemostatic parameters and their relation to risk of diseases such as thrombosis and coronary artery disease.1 Although there is great interest in assessing genetic components of phenotypic variability in hemostasis and its relation to thrombosis,2 most current work has focused on evaluating the role of structural candidate genes through population-based association studies.3,4 Such approaches invariably underestimate the importance and complexity of genetic factors because of their reliance on linkage disequilibrium.5 Comparatively few studies6–8 have attempted to quantify the nature and extent of genetic determinants of phenotypic variation in hemostatic parameters through the use of family-based sampling designs. Such knowledge is critical to inform future genome-wide linkage studies to localize novel regulatory loci involved in coagulation and fibrinolysis.

Given the continuous nature of most commonly assayed hemostasis-related phenotypes, it is likely that there will be a number of interacting genetic and environmental factors that jointly determine their variable expression. Powerful new analytical methods have been developed that ultimately will be used to localize and evaluate the relative effects of these quantitative trait loci (QTLs).9,10 Before such costly analyses, it is necessary to determine which phenotypes can be pursued profitably through linkage studies. Therefore, the primary purpose of this investigation was to examine the roles of genetic and environmental factors in determining hemostasis-related phenotypes. We studied a sample of extended Spanish kindreds, half of which were ascertained through individuals with thrombophilia. This study is the first large-scale family study of the genetics of quantitative variation in these putative risk factors for thrombosis and ischemic heart disease.
METHODS

Enrollment of Family Members
Recruitment of family members was based in Barcelona and was performed as part of the GAIT project. The sample included 21 families selected primarily for pedigree size to maximize the power to detect genetic effects. To be included, a family had to have ≥10 living individuals in ≥3 generations. Twelve families were selected through a proband with idiopathic thrombophilia, which was defined as multiple thrombotic events (≥1 spontaneous), a single spontaneous episode of thrombosis with a first-degree relative also affected, or onset of thrombosis before age 45 years. Ten of the 12 probands had onset before age 45 years, 8 had multiple thromboses, and only 2 were ascertained because of a single episode of thrombosis with a relative also affected. The proband’s thrombophilia was considered idiopathic because all known (during the recruitment period of 1995 to 1997) biological causes (eg, antithrombin deficiency, protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, HCII deficiency, Factor V Leiden, dysfibrinogenemia, lupus anticoagulant, and antiphospholipid antibodies) of thrombophilia were excluded. These thrombophilic factors were also absent in all affected relatives. The remaining 9 families were selected without regard to phenotype.

Subjects were interviewed by a physician to determine their health/reproductive history, current medications, including use of oral contraceptives, and smoking history. They were questioned about 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. The residence of each subject was determined to assess the contribution of shared environmental influences (such as diet) common to members of a household. All procedures were reviewed by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau. Adult subjects gave informed consent for themselves and for their minor children.

A total of 397 individuals were examined, with a mean of 19 individuals and 7 households per family. Subjects ranged in age from <1 year to 88 years, with a mean of 37.7 and approximately equal numbers of male (46%) and female (54%) subjects. Table 1 lists the number of individuals examined by sex for each pedigree as well as the number of additional unexamined family members (most deceased) required to account for biological links among pedigree members. Of the individuals examined, 101 were founders (individuals whose parents are not in the pedigree) and 296 were nonfounders. The number of households per pedigree ranged from 4 to 14 and the number of examined individuals per household ranged from 1 to 7, with a mean of 2.6. Most pedigrees contained 3 generations, with 8 families having 4 generations and 1 having 5. The depth and complexity of these pedigrees is illustrated by the number of relative pairs contained therein (Table 2).

Blood Collection
Blood was obtained by venipuncture after a 12-hour fast. Samples for hemostatic tests were collected in 1:10 volumes of 0.129 mol/L sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000g for 20 minutes at room temperature (22±2°C). Assays for APTT, prothrombin time, and coagulation factors were performed on fresh plasma samples. The remaining plasma samples were stored at −80°C until use. Samples for homocysteine determination were collected in EDTA and kept on ice until plasma was harvested by centrifugation. DNA extraction and storage were performed according to standard protocols.11

Phenotype Assays
APTT and PT were measured in an automated coagulometer (ACL 3000; IL) with the use of bovine cephalin and silica for APTT (IL) and human thromboplastin for PT (Thromborel S; Behring). Fibrinogen, coagulation factors, funcPS, and APCR were assayed in the STA automated coagulometer (Boehringer Mannheim). Fibrinogen was measured by the von Clauss method12 with thrombin from BioMerieux (Marcy-l’Etoile). FII, FV, FVII, FVIII, FIX, FX, FXI, and FXII were assayed with deficient plasma from Diagnostica Stago (Asnières). funcPS was determined with a kit from Diagnostica Stago. APCR was measured with the kit Coatest APC Resistance from Chromogenix. AT, protein C, HCII, plasminogen, and PAI-1 were measured in a biochemical analyzer (CPA Coulter, Coulter Corp) with the use of chromogenic methods from Chromogenix for AT, protein C, and plasminogen and from Diagnostica Stago for HCII and PAI-1.

tPS and tPS, TPA, and DD were assayed with the use of ELISA methods from Diagnostica Stago. TF was tested by an ELISA method from American Diagnostica. von Willebrand factor was measured by an ELISA method with antibodies from Dako. Levels of histidine-rich glycoprotein were measured by electroimmunoassay with antibodies from Diagnostica Stago. TFPI was measured by a functional method as described by Sandset et al.13 Basal homocysteine was separated by HPLC and determined by a fluorometric method.14

To reduce measurement error, assays were performed in duplicate, and the average value was calculated for each person. Intra-assay and interassay coefficients of variation were generally estimated to be between 2% and 6%. However, the interassay coefficients of variation were somewhat higher for DD (16.7%), TFPI (9.7%), and TPA (9%).

Statistical Methods
The goal of these analyses was to determine the contributions of genes, measured environmental factors specific to an individual, and environmental factors shared in common by members of a household to variation in hemostasis-related phenotypes. The phenotypic covariance among relatives was used to estimate the additive genetic and shared environmental components of variance.
The level of a trait, $y_i$, for individual $i$ is modeled as a linear function as follows:

$$y_i = \mu + \sum \beta_j x_{ij} + g_i + h_i + e_i,$$

where $\mu$ is the trait mean in male subjects, $x_{ij}$ is the $j$-th covariate, and $\beta_j$ is its regression coefficient. Covariates included female sex, sex-specific age and age squared, smoking, and for female subjects, current use of oral contraceptives. Age-related covariates were scaled such that the regression coefficients represent the effect associated with a 10-year deviation from the mean age. Discrete covariates (female sex, smoking, and oral contraceptive use) were scaled so that the regression coefficients represent the effect of presence of the covariate versus absence. The remaining variables in the above formula, $g_i$, $h_i$, and $e_i$, represent the random deviations from $\mu$ for individual $i$ that are attributable to additive genetic, household, and residual error effects, respectively. The residual error component includes true random error, measurement error, and any nonadditive genetic components. The effects of $g_i$, $h_i$, and $e_i$ are assumed to be uncorrelated with one another and normally distributed with mean zero and variances $\sigma_g^2$, $\sigma_h^2$, and $\sigma_e^2$. The likelihood of the phenotypes of the family members is assumed to follow a multivariate normal distribution with a phenotypic covariance matrix that is a function of kinship between individuals and the additive genetic, household, and environmental variances.

This approach can be viewed intuitively as decomposing the observed phenotypic correlations among different classes of relatives in terms of underlying genetic and shared environmental factors. Once the expected means and covariance matrix of each pedigree are defined, the likelihood of a pedigree is evaluated with the multivariate normal distribution. Although we assume multivariate normality, this assumption is robust, and consistent parameter estimates are obtained when the assumption is violated.

Because 12 pedigrees were ascertained through a thrombophilic proband, we performed an ascertainment correction to obtain unbiased parameter estimates relevant to the general population. This was achieved by conditioning on the probands’ phenotype. Two pedigrees were ascertained through a thrombophilic proband and an affected relative. Ascertainment correction with both individuals did not produce different results than correction on the focal proband alone. Although ascertainment was based on thrombophilia, our ascertainment correction was performed by conditioning on the hemostasis-related phenotype being analyzed. This conservative correction can lead to larger standard errors of parameter estimates but protects against type I error.

Maximum likelihood methods were used to simultaneously estimate mean and variance values as well as the effects of covariates, heredity, and household through the use of the computer package SOLAR. The significance of covariate effects was assessed with a Wald test. The relative proportions of the residual variance in a trait explained by genetic and household determinants were calculated as the variance attributable to that component divided by the residual phenotypic variance after adjustment for covariates. The significance of genetic and household effects was assessed by comparing the likelihoods of models in which these parameters were estimated to models in which they were constrained to zero. Twice the difference in ln-likelihood between these models is asymptotically distributed as a 1/2:1/2 mixture of $\chi^2_1$ and $\chi^2_3$.

### Results

Regression coefficients for the environmental covariates are shown in Table 3. Sex and age effects were significant for most traits examined. For example, Factor (F)V, FVII, FVIII, FXI, von Willebrand factor, dimer (DD), tissue factor (TF), protein C, TF pathway inhibitor (TFPI), fibrinogen, total protein S (tPS), free protein S (fPS), homocysteine, and tissue plasminogen activator (TPA) showed dramatic increases with age, whereas antithrombin (AT), prothrombin time (PT), and activated partial thromboplastin time (APTT) showed substantial decreases. Similarly, several traits showed significant sex differences, with female subjects generally showing lower age-corrected phenotypic values than male subjects. This is true for the protein S traits, FV, FIX, FX, activated protein C resistance (APCR), homocysteine, TPA, and plasminogen activator inhibitor-1 (PAI-1), for which female subjects have substantially lower mean values than male subjects. In contrast, female subjects exhibited significantly higher levels of FVIII than did male subjects.

Smoking significantly increased levels of heparin cofactor II (HCII) but decreased FV, FVII, FVIII, protein C, and PT. Oral contraceptive use significantly increased FII, FX, HCII, and plasminogen and decreased levels of FVIII, PAI-1, and fPS.

Table 4 presents the estimated components of variance for the hemostasis-related phenotypes. Components of variance are shown for the most parsimonious model (i.e., the model that best fits the observed data and exhibits the minimum of complexity) for each phenotype, including only significant sources of variation. The remaining variance not accounted for in Table 4 is attributable to individual-specific random environmental influences and random error. All of the traits studied except DD had significant genetic components, with most ranging between 22% and 55% of the residual phenotypic variability. APTT, APCR, and FXII showed exceptionally large genetic influences, accounting for 83%, 71%, and 67% of residual variance, respectively. In contrast, DD showed no significant heritable component, with an estimated heritability of 10.9% ($p = 0.07$).

The proportion of the residual phenotypic variance accounted for by shared household effects tended to be considerably smaller than that accounted for by genetic effects. Household components were significant for only 8 traits: tPS, functional protein S (funcPS), FV, FX, FIX, fibrinogen, PAI-1, and fPS. Household membership accounted for $\approx 10\%$ to 16% of the residual phenotypic variability in most of these traits, with only fPS having household effects accounting for $\geq 20\%$ of its residual phenotypic variance.

Likelihood-based tests of heterogeneity allowing for ascertainment correction revealed no differences between ran-
domly ascertained families and families ascertained through thrombophilic probands. This result suggests that the ascertainment correction used was successful in recovering population-based estimates of both covariate effects and the relative variance components.

**Discussion**

Our results document the importance of genetic factors influencing hemostasis-related phenotypes in this population. For most of the traits, genes appear to be the largest identifiable determinant of quantitative variation. The use of extended pedigrees and household-sharing information yielded precise information on the determinants of correlations among family members. Shared environment had a substantial effect on a few phenotypes and was most apparent for fPS. These hemostasis-related phenotypes are similar to other cardiovascular risk factors such as lipoprotein phenotypes, in which shared environmental effects also appear to be of minor importance.

We have limited the estimation of genetic components to that attributable to additive effects. If other nonadditive sources of genetic variance exist, such as dominance or epistasis, then our observed heritabilities will represent lower bounds. Therefore, our estimates are conservative.

Heritability can be diminished by measurement error. One way to increase the genetic signal-to-noise ratio is to eliminate measurement error. In general, the measures considered have modest measurement errors, with interassay and intra-assay coefficients of variation from 2% to 6%. The measurement error for DD is larger (16.7%) and may have contributed to its low observed heritability. However, measurement error of this magnitude is likely to have only a small effect on heritability. If measurement error were eliminated for DD by multiple measures, the estimated heritability would increase only slightly from 0.109 to 0.129. However, the complete elimination of measurement error is not feasible in large studies.

In this study, we have statistically controlled for the effects of demographic and exogenous covariates such as smoking behavior. We have consciously avoided the use of biological covariates that may be influenced by genes. For example, a composite phenotype such as APCR is influenced by a number of intermediate traits such as protein C, protein S, and FVIII. If we were to correct our APCR phenotype for these
correlated phenotypes, the relative genetic and environmental components would be altered unpredictably. Such purely phenotypic correction cannot disentangle genetic correlates from environmental correlates.

We expect that the same genes influence multiple phenotypes. Such pleiotropy is widespread in highly coordinated physiological systems such as coagulation/hemostasis. Additionally, the hemostasis-related phenotypes are correlated with phenotypes from other physiological systems such as the lipid pathway. We do not correct for such covariation because of the potential to eliminate genetic signals that may be important for mapping QTLs that influence hemostasis. For example, it is conceivable that a locus could influence both a hemostasis-related phenotype and a lipid trait. Only joint analysis of both traits could unequivocally determine whether the locus acts through direct effects on each phenotype.

TABLE 4. Components of Variance From the Most Parsimonious Model ± SE

<table>
<thead>
<tr>
<th>Variable</th>
<th>Heritability</th>
<th>Household</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT</td>
<td>0.830±0.067</td>
<td></td>
</tr>
<tr>
<td>APCR</td>
<td>0.713±0.078</td>
<td></td>
</tr>
<tr>
<td>FXII</td>
<td>0.673±0.085</td>
<td></td>
</tr>
<tr>
<td>FVII</td>
<td>0.523±0.089</td>
<td></td>
</tr>
<tr>
<td>Histadine-rich glycoprotein</td>
<td>0.522±0.093</td>
<td></td>
</tr>
<tr>
<td>TFPI</td>
<td>0.516±0.086</td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>0.504±0.085</td>
<td></td>
</tr>
<tr>
<td>Protein C</td>
<td>0.501±0.086</td>
<td></td>
</tr>
<tr>
<td>FII</td>
<td>0.492±0.088</td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>0.486±0.086</td>
<td></td>
</tr>
<tr>
<td>tPS</td>
<td>0.460±0.088</td>
<td>0.108±0.057†</td>
</tr>
<tr>
<td>funcPS</td>
<td>0.453±0.096</td>
<td>0.095±0.060†</td>
</tr>
<tr>
<td>FXI</td>
<td>0.452±0.104</td>
<td>0.162±0.078†</td>
</tr>
<tr>
<td>FV</td>
<td>0.442±0.094</td>
<td>0.133±0.067†</td>
</tr>
<tr>
<td>HCl</td>
<td>0.439±0.086</td>
<td></td>
</tr>
<tr>
<td>FX</td>
<td>0.434±0.127</td>
<td>0.135±0.076†</td>
</tr>
<tr>
<td>FVIII</td>
<td>0.400±0.088</td>
<td></td>
</tr>
<tr>
<td>FIX</td>
<td>0.387±0.086</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.336±0.101</td>
<td>0.137±0.065†</td>
</tr>
<tr>
<td>von Willebrand Factor</td>
<td>0.318±0.108</td>
<td></td>
</tr>
<tr>
<td>PAI-1</td>
<td>0.298±0.080</td>
<td>0.139±0.061‡</td>
</tr>
<tr>
<td>TPA</td>
<td>0.268±0.072</td>
<td></td>
</tr>
<tr>
<td>Homocysteine</td>
<td>0.244±0.077</td>
<td></td>
</tr>
<tr>
<td>Plasminogen</td>
<td>0.236±0.096‡</td>
<td></td>
</tr>
<tr>
<td>fPS</td>
<td>0.223±0.106‡</td>
<td>0.212±0.065§</td>
</tr>
<tr>
<td>TF</td>
<td>0.167±0.079‡</td>
<td></td>
</tr>
<tr>
<td>DD</td>
<td>0.109±0.091*</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.10, †P < 0.05, ‡P < 0.01, §P < 0.001, ††P < 0.0001

pleiotropy will require multivariate genetic analysis, and any correlation between traits, whether caused by genes or environment, can be exploited to increase the power of a subsequent linkage study.9

The utility of genetic studies of quantitative intermediate risk factors is manifold. Intermediate risk factors are more proximal to gene action and thus provide less attenuated genetic signals than when a discrete clinical end point such as disease is analyzed. Also, susceptibility to disease is primarily a quantitative process that reflects an unobservable continuous liability. Evidence for the continuous relation between several of the hemostasis-related risk factors considered in this study and risk of venous thrombosis has been widely reported. For example, APCR shows an inverse continuous relation with risk of thrombosis,18 whereas fibrinogen,19 FVIII,20 FII,21 and homocysteine levels22 all exhibit continuous positive relations with risk of thrombosis. Liability to thrombosis is influenced not only by abnormalities in these systems but also by quantitative variation within the normal physiological range. Such candidate risk factors can be utilized jointly with disease status to search the genome for QTLs that pleiotropically affect both risk factor and disease.

A primary goal of modern genetic analysis is to partition the genetic variability in a phenotype into components attributable to specific QTLs. Such goals can now be attained with the use of powerful new methods of quantitative trait linkage analysis on human pedigree data such as that collected for the Genetic Analysis of Idiopathic Thrombophilia (GAIT) study. These new linkage approaches will provide estimates of chromosomal location and, equally important, unbiased estimates of the relative importance of specific QTLs for the general population. Such estimates will be essential for the decomposition of the risk of disease in the general population and therefore are relevant to public health. Ultimately, the joint analysis of both thrombosis and its quantitative risk factors will lead to the identification of the genes determining risk of thrombosis. Such information then may be used for predicting individual-specific risk early enough in life to consider prophylactic intervention.

Candidate gene studies can provide some information regarding the likelihood of finding novel QTLs by linkage analysis. For example, data provided by de Ronde and Bertina23 on the FV Leiden mutation suggest that 34% of the phenotypic variance in APCR in the Netherlands may be attributable to this gene. However, even considering the lower-bound nature of association-derived locus-specific effects,7 it is unlikely that such genes account for all or most of the variability in the quantitative risk factors considered. The FV Leiden mutation results have particular relevance for the current study. Since this mutation is much rarer in the Spanish population,24 it could account for little (<5%) of the variation in APCR in our sample, yet our estimated heritability for the Spanish population is very high (71%). Although some of this genetic variance may be attributable to unknown mutations in the FV gene, it is likely that some of it is attributable to other unknown genes. If some of these novel genes exhibit comparable effects on the hemostasis-related phenotypes as those seen in the candidate gene studies, it should be relatively easy to localize them in linkage-based designs whose power
depends solely on the relative heritability attributable to the QTL. To this end, our results showing substantial total heritabilities for most of the measured hemostasis-related phenotypes provide excellent support for our plan to perform a genomic search to identify and assess the importance of these genes in the Spanish population.

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