5.6 Efectos funcionales del polimorfismo en el *locus* ABO sobre los niveles en plasma del factor von Willebrand, factor VIII y el tiempo de tromboplastina parcial activado

(*Arterioscler Thromb Vasc Biol 2000;20:2024-2028*)

Functional Effects of the ABO Locus Polymorphism on Plasma Levels of von Willebrand Factor, Factor VIII, and Activated Partial Thromboplastin Time

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Abstract—Lower levels of factor VIII and von Willebrand factor (vWF) have been reported in individuals with blood type O compared with individuals with other ABO blood types. However, this relationship has been demonstrated only by association studies and not by linkage studies. Also, it is not clear whether the ABO locus exerts a functional effect directly on these plasma factors or whether the ABO locus is in linkage disequilibrium with another locus that controls these factors. To distinguish between these 2 possibilities, we applied new statistical methods combining linkage and association tests in a pedigree-based sample. In contrast to most previous studies that used the ABO phenotypes, our study used the ABO genotypes, permitting us to distinguish AO from AA and BO from BB. Our results clearly showed significant linkage between the ABO locus and vWF antigen ($P=0.00075$). In addition, factor VIII coagulant activity and activated partial thromboplastin time showed suggestive linkage with the ABO locus ($P=0.10$ and $P=0.13$). All 3 plasma phenotypes showed significant differences between OO and non-OO genotypes. In addition, vWF antigen exhibited significant differences between O heterozygotes and non-OO homozygotes. This study is unique because it used a combined linkage and association test, which indicated that the ABO locus itself has a functional effect on these plasma phenotypes. **(*Arterioscler Thromb Vasc Biol*. 2000;20:2024-2028.)**

Key Words: ABO blood group ■ von Willebrand factor ■ factor VIII ■ genetics ■ functional polymorphism

Since the 1960s and the early 1970s, many studies have reported a relationship between the ABO blood group and the risk of coronary heart disease,[1,2] atherosclerosis,[3,4] and venous thromboembolic disease.[1,5] In addition, >15 reports have established an association between the ABO blood group and plasma levels of factor VIII[6–9] and von Willebrand factor (vWF).[6–8,10–12] A relationship between some global coagulation tests, such as activated partial thromboplastin time (APTT), and ABO phenotypes has also been reported.[9,13]

It is well known that a relationship exists between vascular diseases and vWF or factor VIII and that vWF is related to venous thromboembolism,[5,14–16] cerebral arterial disease,[14,17] and coronary heart disease.[2,18–20] Factor VIII is also associated with coronary risk,[2,20] ischemic cerebrovascular disease,[17] and venous thrombotic disease.[5,16] Our own data from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Project[21] support these results and unequivocally demonstrate that factor VIII and vWF are genetically correlated with thrombotic risk. In other words, there are some genes with multiple (pleiotropic) effects that simultaneously influence the risk of thromboembolic disease and the plasma levels of these proteins.

Notably, all of the studies cited above found an increased risk of disease, as well as higher plasma levels of factor VIII and vWF, for all of the ABO phenotypes except type O.[1,2,6,8,9,11] In fact, type O individuals seem to show lower coagulability than A, B, or AB individuals.[9,13] The majority of the evidence for correlations between ABO blood group, plasma factors, and disease stems from classic association studies, most of them retrospective case-control investigations. Thus, no unequivocal causal relationships have been established between ABO and plasma factor VIII/vWF levels and the development of vascular disease. In addition, it is impossible to state whether the associations between ABO blood type and levels of factor VIII or vWF are due to a functional effect of the ABO locus or whether ≥1 allele of the ABO locus, particularly type O, is in linkage disequilibrium with a functional polymorphism at a linked site. Linkage disequilibrium is a population-wide nonrandom association of alleles at 2 syntenic sites. It occurs initially by the appearance of a new mutation on a particular genetic background (haplotype). Normally, it decays with time through recombination.

Monogenic forms of vWF deficiency (von Willebrand disease) and factor VIII deficiency (hemophilia A) have been

Received October 19, 1999; revision accepted January 21, 2000.

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***Arterioscler Thromb Vasc Biol.* is available at http://www.atvbaha.org**

linked to the structural loci on human chromosome 12[22] and the X chromosome,[23] respectively. There are no linkage studies of normal variability in plasma levels of APTT, factor VIII, or vWF. We have recently demonstrated that these phenotypes have significant genetic components.[24] For example, factor VIII coagulant activity (factor VIII:C) had an additive genetic heritability of 0.40, whereas the heritability of vWF antigen (vWF:Ag) was 0.32 and that of APTT was 0.83. The above-mentioned epidemiological studies suggest that ABO may be one of the loci underlying these genetic effects. However, because all of the previous studies were population-based case-control studies, they were susceptible to false-positive associations due to hidden stratification. To obviate this difficulty, we sought to confirm the putative relationship between ABO and the plasma factors by using the more definitive genetic linkage methods. Although most previous studies have used phenotypic assays of ABO blood types, in which AA and BB homozygotes were indistinguishable from AO or BO heterozygotes, our analyses were based on molecularly defined ABO genotypes. In addition, we applied new powerful statistical methods that simultaneously test for linkage and for association and can distinguish between functional polymorphisms and linkage disequilibrium.

Methods

Study Population

For the present study, we used DNA and plasma samples from the pedigreed families belonging to the GAIT Project. Our sample was composed of 21 extended families, 12 of which were ascertained through a proband with thrombophilia and 9 of which were obtained randomly from the general population. Thrombophilia was defined as 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. Diagnoses of the 12 thrombophilic probands were verified by objective methods. Thrombosis in these individuals was considered idiopathic because all of the biological causes of thrombosis known at the time of recruitment (1995 to 1997) were excluded. These included 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.

We examined 397 individuals, with an average of 19 individuals per family. Most of the pedigrees contained 3 generations, with 8 families having 4 generations and 1 family having 5 generations. The mean age at examination of the individuals was 37.7 years, and the proportion of males and females was approximately equal. The composition of the families and the collection of lifestyle, medical, and family history data are detailed in Souto et al.[24] The history of thrombosis in family members was verified by examination of medical records. Although some deceased family members were recorded as affected, only individuals interviewed and examined in person were included in the analyses. The study was performed according to the Declaration of Helsinki of 1975, and all adult patients provided informed consent for themselves and for their minor children. All procedures were reviewed by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau.

Laboratory Measurements

Blood was obtained from the antecubital vein after the subject had fasted for 12 hours. Samples for hemostatic tests were collected in 1/10 vol of 0.129 mol/L sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000*g* for 20 minutes at room temperature (22±2°C). Assays for APTT and factor VIII:C were performed immediately on fresh plasma samples. The remaining plasma samples were stored at −80°C until use.

APTT was measured in an automated coagulometer (ACL 3000, IL) with the use of bovine thromboplastin and silica (APTT-silica, IL). The control sample consisted of IL-test calibration plasma. A functional assay for factor VIII activity was obtained by using the automated coagulometer STA (Diagnostica Stago) with use of deficient plasma from Diagnostica Stago and APTT-silica from IL. The Unicalibrator standard from Diagnostica Stago was used and was calibrated with the international standard for factor VIII. vWF was measured by ELISA with use of polyclonal antibodies from Dako. APTT was quantified as a ratio of thromboplastin time relative to a pooled-plasma control, whereas factor VIII:C and vWF:Ag were recorded as percentages of an international standard sample. To reduce measurement errors, all assays were performed in duplicate, and the average value was calculated for each person. The interassay coefficients of variation were within accepted levels for all 3 measures (3.8% for APTT, 4.2% for factor VIII:C, and 6.0% for vWF:Ag).

Blood Group Serology and DNA Preparation

Samples were phenotyped by routine erythrocyte and serum blood grouping procedures.

DNA was extracted from EDTA blood by use of a salting-out method[25] or a commercial kit (Wizard, Promega Corp). ABO genotyping was performed by polymerase chain reaction (PCR) as described[26] with use of the following primers: for exon VI, mo-46 5′-CGGAATTCACTCGCCACTGCCTGGGTCTC-3′ and mo-57 5′-CGGATCCATGTGGGTGGCACCCTGCCA-3′; and for exon VII, mo-101 5 ′-CGGGATCCCCGTCCGCCTGCCTTGCAG-3′ and mo-71 5′-GGGCCTAGGCTTCAGTTACTC-3′.

PCR was performed in a total volume of 10 μL under the following conditions: initial denaturation at 94°C for 2 minutes, then 10 cycles at 94°C for 10 seconds, followed by annealing and extension at 65°C for 60 seconds, and finally 20 cycles of denaturation at 94°C for 10 seconds, annealing at 61°C for 50 seconds, and extension at 72°C for 20 seconds. For each reaction, 2 pmol of each primer was mixed with 0.1 μg genomic DNA and 0.5 U of *Taq* polymerase (Perkin-Elmer Cetus) in the commercial buffer.

The amplified DNA was digested with a mixture (5 μL) containing 2 U each of restriction endonucleases *Hpa*II and *Kpn*I (GIBCO Life Technologies) in a 3× concentrated buffer (NEBuffer 4), 200 mmol/L Tris-acetate, 100 mmol/L magnesium acetate, 500 mmol/L potassium acetate, and 10 mmol/L dithiothreitol, pH 7.9 (New England Biolabs). Digests were incubated for 2 hours by use of the GeneAmp PCR System 9600 at 37°C. Cleavage products were separated electrophoretically for 1 hour at 100 V (10 V/cm) in an EC-105 (E-C Apparatus Corp) and visualized by using 4% agarose gels containing 0.56 mg ethidium bromide from Sigma Chemical Co.

Statistical Genetic Analysis

Pedigree-based variance component linkage analyses were performed by use of the SOLAR program.[27] This method uses the correlation of phenotypes between relatives to partition the variance in the trait 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. Information on genome-wide additive genetic effects on the variance (ie, heritability) comes from the kinship between family members, whereas linkage information regarding specific quantitative trait loci comes from estimates of the proportion of alleles shared identically by descent between individuals for each genetic marker tested. Sex and sex-specific age and age-squared were included as covariates in all analyses. Bivariate analyses, which use the correlations between phenotypes to test hypotheses of pleiotropy and to improve the power to detect linkage, were performed with a modified version of SOLAR.[28]

The SOLAR program was also used to test for genotype-specific differences in trait means (measured genotype analyses) while taking into account the family structure of the data.[29] To assess linkage and association simultaneously, linkage tests were performed incorporating the genotype-specific means of the measured genotype test. If a variant is the only functional polymorphism in a chromosomal region, the measured genotype test provides all of the genetic information, and the linkage test provides no additional information.

TABLE 1. ABO Distribution According to Phenotype and Genotype in 391 GAIT Individuals

ABO Phenotype	Gross Genotype	Specific Genotype	n
Group O (N=139)	OO	O_1/O_1	131
		O_1/O_2	8
		O_2/O_2	0
Group A (N=211)	AO	A_1/O_1	151
		A_1/O_2	0
		A_2/O_1	12
		A_2/O_2	0
	AA	A_1/A_1	33
		A_1/A_2	14
		A_2/A_2	1
Group B (N=31)	BO	B/O_1	30
		B/O_2	1
	BB	B/B	0
Group AB (N=10)	AB	A_1/B	10
		A_2/B	0

Therefore, the logarithm of the odds ratio (LOD) score should drop to zero. Alternatively, if there are other nearby functional sites or if a variant is merely in linkage disequilibrium with a functional site, linkage analyses will have additional predictive power over the measured genotype test. In other words, the LOD score will not drop to zero.

Variance component parameters were estimated through maximum-likelihood methods, and the hypotheses were tested by use of likelihood-ratio test statistics.[30,31] Because 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.[29,32]

Results

Linkage analyses were performed with the use of ABO genotypes (Table 1), which distinguish the O_1 and O_2 alleles and the A_1 and A_2 alleles as well as A/O and B/O heterozygotes. Significant evidence of linkage was observed between the ABO locus and the vWF:Ag plasma levels, with a LOD score of 2.19 ($P=0.00075$). In addition, factor VIII:C and APTT exhibited weak evidence of linkage, with LOD scores of 0.35 ($P=0.10$) and 0.26 ($P=0.13$), respectively. Significantly, a bivariate linkage analysis of vWF:Ag and factor VIII:C with ABO provided strong evidence for a high genetic correlation between the locus-specific effects on the 2 traits ($P=0.0005$). This suggests that either the ABO locus itself or a locus linked to it acts pleiotropically to jointly affect both phenotypes.

Because of the relatively low frequency of the A_2 and O_2 alleles, we grouped alleles A_1 and A_2 into A and O_1 and O_2 into O for the association analyses. Table 2 shows the mean phenotypic values by ABO genotypes, unadjusted for covariates or familial relationships. Sample sizes differ among phenotypes because of inadequate plasma volumes for ≈60 individuals. The lowest mean values of vWF:Ag (77.3±27.4%) and factor VIII:C (131.8±47.1%) were observed with type O, and the highest vWF:Ag values (136.7±33.7%) and factor VIII:C values (170.9±60.1%) corresponded to type AB. The highest mean APTT value (0.977±0.103) was found among the O type, whereas the lowest was among the AB type (0.879±0.068). Measured genotype analyses were conducted allowing different trait means for O homozygotes (35.5%), O heterozygotes (49.6%), and individuals with no O alleles (14.8%). All 3 phenotypes showed significant differences between O homozygotes and the other genotypes (vWF:Ag, $P=1\times10^{-7}$; factor VIII:C, $P=8.2\times10^{-6}$; and APTT, $P=0.001$). However, only vWF:Ag showed significantly lower levels in heterozygous carriers of an O allele (A/O and B/O) versus noncarriers of the O allele (A/A, A/B, and B/B; $P=0.03$). These association results provide strong evidence for a locus at or near the ABO gene influencing these phenotypes.

TABLE 2. Comparison of Values of vWF:Ag, Factor VIII:C, and APTT With the ABO Genotypes

Genotype	vWF:Ag, %	Factor VIII:C, %	APTT Ratio
O/O	77.3±27.4	131.8±47.1	0.977±0.103
A/O	113.8±40.8	162.1±52.5	0.934±0.097
B/O	102.8±30.2	155.5±55.1	0.940±0.105
A/A	118.0±39.7	164.3±50.5	0.947±0.105
A/B	136.7±33.7	170.9±60.1	0.879±0.068
No. of tested individuals	328	387	389

Values are mean±SD.

To further refine our inferences, we performed a combined linkage/association analysis. The primary goal of this analysis was to determine whether the ABO genotype itself could completely account for our observed linkage. If explicitly controlling for the ABO genotype eliminates all evidence of linkage, then the hypothesis that the ABO genotype is itself the functional variant responsible for the linkage is strongly supported. When the combined linkage/association analysis was performed, incorporating the association with ABO genotype, the previously observed linkage signals completely disappeared (the LOD scores dropped to 0), and the relative variance component associated with the quantitative trait locus (QTL) in this region was estimated at 0. This indicates that the genetic effects of the ABO locus on the levels of vWF:Ag, factor VIII:C, and APTT are most likely due to the pleiotropic effects of the ABO polymorphism itself and not to linkage disequilibrium. Similarly, all of the variance attributed to QTL in this region is absorbed into the ABO genotype. In other words, the ABO genotype contains all necessary information to completely characterize the inferred QTL and most likely represents the functional variant responsible for the genetic signal in this chromosomal region.

Discussion

Although an effect of the ABO locus on vWF and factor VIII levels has long been suggested by association studies, the present study is the first demonstration of linkage between ABO locus and these 2 phenotypes. As mentioned earlier, case-control studies can lead to false-positive associations because of population stratification. For example, in an ethnically mixed population, vWF may be correlated with ABO by virtue of each one's association with ethnicity, with no direct causal connection between them. Linkage analyses are not susceptible to this problem because they examine

cosegregation of genotype and phenotype within families. We observed significant linkage between ABO and vWF:Ag and suggestive linkage with factor VIII:C and APTT. Because vWF is the carrier molecule of factor VIII and because they are functionally closely related, we used bivariate linkage analyses of vWF:Ag and factor VIII:C with ABO to exploit the correlations between phenotypes to improve the power of our linkage analyses.[28] In the bivariate test, we demonstrated a significant genetic correlation between the effects of the ABO locus itself or an ABO-linked locus on vWF:Ag and on factor VIII:C. This suggests that the locus influencing vWF:Ag also affects factor VIII:C, strengthening the suggestive linkage observed between ABO and factor VIII:C.

One of the strengths of the present study is the use of the ABO genotypes rather than ABO phenotypes. Of previous studies, only Shima et al[33] used molecular methods to precisely delineate the ABO genotype in relation to vWF levels. The ability to distinguish the A_1 and A_2 alleles and the O_1 and O_2 alleles and the ability to detect A/O and B/O heterozygotes, which were previously indistinguishable, enhances considerably the identity-by-descent information content of the ABO locus and consequently improves the power to detect linkage. In the family-based association analyses, we observed the same differences between the O phenotype and all of the other phenotypes that have been reported previously. However, because we could distinguish individual heterozygotes for the O allele, we were also able to demonstrate a significant difference in vWF:Ag levels between these heterozygotes and noncarriers of the O allele. This relationship was also observed by Shima et al in an association analysis of unrelated individuals.

The present study is notable because it uses the new statistical methods that combine information about linkage and association to distinguish the functional effects of a polymorphism from an association that is due to linkage disequilibrium with a different functional site. Although it is well known that the ABO blood types are associated with vWF and factor VIII, it has been impossible to determine whether the O allele is itself functional with pleiotropic effects on vWF and factor VIII. Alternatively, it was possible that linkage disequilibrium accounted for this association. However, our evidence strongly indicates that the O allele has a direct functional effect on vWF:Ag, factor VIII:C, and APTT, because linkage provided no additional predictive information over association with the O allele in the combined linkage/association tests. However, our results could still be due to an unknown functional variant that is in complete linkage disequilibrium with the ABO locus and that has the exact same allelic frequencies as the ABO locus. This joint requirement of total disequilibrium and identical allelic frequency spectra renders this alternative hypothesis extremely unlikely.

As a glycoprotein, vWF has a great diversity of oligosaccharide structures,[34] including molecules similar to blood group A, B, and H (O) antigens.[35] Some of the functions of the vWF molecule, like platelet agglutinating activity in the presence of ristocetin, susceptibility to proteolytic degradation, and survival in the circulation, seem to be affected by the removal of sugar residues. This suggests that carbohydrates are important in the structure/function relationship of vWF.[11] Perhaps the ABO blood group determinants are affecting the processing or the release or catabolism of vWF. By so doing, they may influence the plasma concentration of this protein and, indirectly, the plasma concentration of factor VIII, which is carried by vWF. On the basis of the established relationships between ABO blood group, factor VIII, vWF, and vascular diseases, this hypothesis can be carried one step further. If, as suggested, the ABO blood group and the increased level of vWF heighten thrombotic risk, then the ABO blood group may be only indirectly related to risk of disease through its influence on the plasma levels of vWF.

Thrombosis is a multifactorial complex disease, involving the actions of genes and environmental risk factors and their interactions with each other. However, great progress has recently been made in elucidating the relationships between thrombosis and its quantitative clinical risk factors, including vWF:Ag and factor VIII:C.[21] Additionally, studies of candidate genes involved in hemostasis have identified functional polymorphisms that influence the quantitative phenotypes and risk of thrombosis.[36–39] A recent association study has yielded further evidence that the vWF structural locus influences vWF levels.[40] Interestingly, recent work on a mouse model has revealed a modifier locus involving lineage-specific expression of a glycosyltransferase that also influences vWF levels.[41] Such modifiers are expected to be involved in the expression of complex quantitative phenotypes, such as vWF and factor VIII levels. As seen in our results, the ABO locus acts as a QTL for these phenotypes. However, it is likely that even more loci are involved in determining the variation in these important hemostasis-related traits. It is anticipated that complete genome screens will soon localize unidentified loci. In the near future, we will be able to use genotypes to augment the quantitative clinical assays in defining a profile for thrombosis risk in an individual. Our results, combined with those of previous investigators, indicate that ABO phenotyping and, in addition, ABO genotyping may be valuable components of future diagnostic thrombophilia risk profiles and might have implications in the policy of thrombosis prophylaxis and treatment.

Acknowledgments

This study was supported by grants DGICYT Sab 94/0170 from the Ministerio de Educación y Ciencia, Spain; FIS 97/2032 from the Ministerio de Sanidad y Consumo, Spain; RED97/3 from the Generalitat de Catalunya, Spain; and National Institutes of Health grants MH-59490 and GM-18897. We are grateful to a number of doctors who assisted in the ascertainment and recruitment of thrombophilic pedigrees: Dr Javier Rodríguez Martorell from Hospital Universitario Puerta del Mar, in Cádiz; Dr Carmen Araguás from Hospital Arnau de Vilanova, in Lleida; Dr Francisco Velasco from Hospital Reina Sofía, in Córdoba; and Dr Montserrat Maicas from the Hospital General de Albacete and Dr Dilia Brito from Hospital Carlos Haya, in Málaga. We would also like to acknowledge the technical assistance of Pilar Santo Domingo, Marina Arilla, and Neus Boto in performing laboratory assays and the work of Alfonso Buil in the day-to-day operations of data management. Finally, we are deeply grateful to all of the families who have participated in this study.

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5.7 Heredabilidad de fenotipos plasmáticos relacionados con la Hemostasia y estudio de su correlación genética con el riesgo de trombosis: resultados del proyecto GAIT

(sometido a *Arterioscler Thromb Vasc Biol*)

HERITABILITIES OF HEMOSTASIS-RELATED PHENOTYPES AND THEIR GENETIC CORRELATIONS WITH THROMBOSIS: THE GAIT PROJECT

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Sometido para publicación a
Arteriosclerosis, Thrombosis and Vascular Biology

ABSTRACT

The GAIT (Genetic Analysis of Idiopathic Thrombophilia) Project is a family-based study dedicated to elucidating the genetic basis of hemostasis-related phenotypes and thrombosis risk. In this paper, we have examined several lesser-studied hemostasis-related phenotypes in the 21 GAIT families: levels of vitamin B12, serum folate, whole blood folate, α_2-antiplasmin, prekallikrein, β_2-glycoprotein I, soluble P-selectin, Factor XIII A and S subunits and a new coagulation measurement based on thromboplastin time in the presence or absence of thrombomodulin. Using the variance component method, we estimated the relative contributions of genetic and environmental influences on these phenotypes. In addition, we calculated the genetic correlations between thrombosis risk and each of these phenotypes.

All 12 phenotypes showed significant genetic contributions with genes accounting for 22% to 78% of the variance after correction for covariate effects. Four phenotypes (three traits involving thromboplastin-thrombomodulin mediated coagulation time and serum folate) exhibited significant genetic correlations with thrombosis. Thus, some of the genes that influence quantitative variation in these physiological phenotypes also influence the risk of thrombosis.

The high heritabilities and significant genetic correlations between thrombosis and some risk factors suggest that joint consideration of correlated quantitative phenotypes will aid in identifying susceptibility genes.

Key Words: thrombosis, coagulation, genetics, family-based studies, heritability

Plasma levels of hemostasis-related phenotypes, as complex traits, are likely influenced by a number of interacting genetic and environmental factors (1). All of these factors acting jointly explain the variable expression among different individuals. Thrombosis, as with the majority of common diseases, is also complex, and is influenced by both environmental and genetic factors (2). Although disease status is usually characterized as a discrete trait with individuals categorized as unaffected or affected, it is important to recognize that underlying the dichotomous disease categorization is a continuous trait, termed liability, susceptibility or risk. While liability cannot be directly observed or measured, it can be modeled and estimated. If an individual's estimated liability score exceeds the critical threshold, disease results, whereas if it is below the threshold, the individual is unaffected. These threshold models of an underlying continuous scale of disease risk allow inferences that are consistent with current models of gene action (3).

Given the complexity underlying the regulation of plasma levels of hemostasis-related phenotypes and individual thrombosis risk, classical statistical methods, as well as standard epidemiological methods, based on sampling of unrelated individuals, are unable to disentangle and quantify the components (genetic and environmental) responsible for this variability. Fortunately, there are powerful statistical genetic methods that allow the simultaneous examination of the genetic and environmental sources of the correlations between quantitative physiological measures and disease outcome (4) through the analyses of family data. These statistical genetic methods also permit localization and evaluation of the relative effects of the genes involved (5).

We previously have applied these powerful statistical genetic methods in our GAIT Project using extended Spanish kindreds. We estimated that over 60% of the variation in susceptibility to thrombosis is attributable to genetic factors and this represented the first report quantifying the genetic basis of susceptibility to thrombosis (6). In another publication (7), we explored 27 commonly-measured potential risk factors and reported that most of the heritabilities of these hemostasis-related phenotypes ranged between 22% and 55% of the residual phenotypic variance after correction for covariate effects. The highest heritabilities were 83% for activated partial thromboplastin time, 71% for APC resistance and 67% for factor XII. In joint analyses with thrombosis liability, we found that nine of those 27 phenotypes were significantly genetically correlated with thrombosis risk, indicating that some of the genes that regulate the quantitative variation of these phenotypes also influence the risk of thrombosis. These phenotypes were factors VII, VIII, IX, XI and XII, von Willebrand factor, t-PA, homocysteine and APC resistance (6).

Given our previous success relating the genetic basis of plasma risk factors influencing thrombosis, we decided to extend our observations to another set of 12 less commonly measured phenotypes related to hemostasis. We used the GAIT families and applied the same statistical methods that we previously utilized. All of these phenotypes exhibited highly significant heritabilities and, more importantly, some of them appear to be genetically correlated with thrombosis risk.

METHODS

Enrollment of Family Members

Recruitment of family members took place between January 1995 and April 1997. The sample included 21 families selected primarily for pedigree size to maximize power to detect genetic effects. To be included, a family had to have at least 10 living individuals in three or more generations. Twelve families were selected through a proband with idiopathic thrombophilia which was defined as multiple thrombotic events (at least one spontaneous), a single spontaneous episode of thrombosis with a first-degree relative also affected, or onset of thrombosis before age 45. Ten of the twelve probands had onset before 45 years, eight experienced multiple thromboses, and only two were ascertained because of a single episode of thrombosis with an affected relative. The proband’s thrombophilia was considered idiopathic because all known (during the recruitment period) biological causes (e.g., antithrombin deficiency, Protein S and C deficiencies, activated protein C resistance, plasminogen deficiency, heparin cofactor II deficiency, Factor V Leiden, dysfibrinogenemia, lupus anticoagulant, and antiphospholipid antibodies) of thrombophilia were

excluded. These thrombophilic factors were also absent in all of the affected relatives. The remaining 9 families were selected without regard to their phenotypes.

The family members were interviewed by a physician to determine their health/reproductive history, current medication usage including 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 recorded to assess the contribution of environments (such as diet) shared by members of a household. A total of 398 individuals were examined. More detailed information about the demographic characteristics of the sample can be found in Souto *et al* (7). Details regarding thrombotic events (e.g., localization, age at event, type of thrombosis, etc.) are available elsewhere (6). This study was reviewed and approved 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.

Blood Collection

Blood was obtained by venipuncture after a 12-hour fast. Samples for hemostatic tests were collected in 1/10 volume of 0.129M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000g for 20 minutes at room temperature (22±2 °C) and stored at –80 °C until used. Samples for the determination of vitamins related to homocysteine metabolism were collected in serum and also frozen at –80 °C until determinations were performed. Control plasma was prepared with a pool of plasmas from 80 healthy blood donors.

Phenotype Assays

Serum vitamin B12 was tested using a commercial kit (ACS Ciba-Corning, catalogue # LKF01). Serum folate and whole blood folate were determined using a commercial kit (Immulite, DPC, catalogue # 672211) and following the instructions of the manufacturer. The determinations of α_2-antiplasmin and prekallikrein were done by means of functional methods in a biochemical analyzer (CPA Coulter, Coulter Corporation, Miami, FL) using chromogenic methods (Coamatic Plasmin-Inhibitor for α_2-antiplasmin and Plasma Prekallikrein Activator and the substrate S2302 for prekallikrein) from Chromogenix, Mölndal. The β_2-glycoprotein I was measured by an antigenic method (Liatest Beta2GP, from Boheringer Mannheim, Mannheim). Soluble P-selectin (sP-selectin) was assayed by an ELISA method from R&D Systems (Minneapolis, MN). Antigenic levels of Factor XIII A and S subunits were determined by a Laurell method using commercially supplied antibodies (Assera XIII-A and XIII-S, Boheringer Mannheim, Mannheim)

As a novel measure of procoagulant capacity of the plasma, we developed a method that consisted of a measure of the thromboplastin time in presence and in absence of thrombomodulin: "Thromboplastin-thrombomodulin mediated time" (Tp-TM T). The laboratory protocol and operating characteristics of this new approach are described in a currently submitted manuscript (Borrell, unpublished data). Briefly, the procedure was as follows: 100 μL of the assayed plasma diluted 1/10 was mixed at 37 °C with 100 μL of a solution (reagent A) containing thromboplastin (Thromborel S; Behring, Marburg) diluted 1/4 and 7.5 U/mL of thrombomodulin (diluted in 0.05% lubrol PX) and the coagulation time was recorded. A second sample of 100 μL of plasma also diluted 1/10 was mixed and assayed at 37 °C with a different solution (reagent B) containing thromboplastin diluted 1/4 and buffer 0.02M Tris, 0.1M of NaCl pH 7.4, 0.05% lubrol PX and 0.02% sodium azide. The results were expressed as:

1) R1 or the ratio between the clotting time of the patient's plasma versus the clotting time of the control plasma using reagent A.
2) R2 or the ratio between the clotting time of the patient's plasma versus the clotting time of the control plasma using reagent B.
3) Finally we obtained R1/R2, the ratio between R1 and R2.

Because of inadequate amounts of plasma samples we were unable to assay all 398 of the GAIT individuals. Thus, serum folate and vitamin B12 were measured in 373 individuals and whole blood folate was assayed in only 151 individuals. The other phenotypes were determined in 315 individuals.

Intra- and inter-assay coefficients of variation were less than 10% for all the phenotypes with the exception of Factor XIII-A (12.1%) and Factor XIII-S (13.9%).

Statistical Methods

1. Components of variance

A major goal of these analyses was to determine the contributions of genes to the variation in the explored phenotypes as well as the percentage of variation due to environmental factors specific to an individual and environmental factors shared in common by members of the same household. The phenotypic covariance among relatives was modeled as described in Souto et al. (7) and was used to estimate the additive genetic and shared environmental components of variance. Covariate effects were estimated simultaneously and included female sex, sex-specific age and age-squared, smoking, and for females, 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 represented the effect of the presence of the covariate versus its absence.

This approach can be viewed intuitively as partitioning the observed phenotypic correlations among relatives with different degrees of relationship in terms of underlying genetic and shared environmental factors.

Maximum likelihood methods were used to estimate simultaneously mean and variance values as well as the effects of covariates, heredity, and household, using the computer package *SOLAR* (5). Likelihood ratio statistics were used to perform hypothesis tests (8, 9).

2. Correlations between quantitative phenotypes and thrombotic risk:

Another goal of our studies was to investigate the genetic relationships between thrombosis susceptibility and quantitative variation in the explored phenotypes. In the pedigree-based, maximum-likelihood method that we used, the presence or absence of thrombosis in a given individual was modeled as a threshold process and was analyzed jointly with the quantitative phenotypes using a mixed discrete/continuous trait variance component technique (4) using the *SOLAR* computer package. This analysis allows the phenotypic correlations between these traits to be partitioned into factors due to common genetic influences and common environmental influences on the two traits. By studying two traits in extended families, we can estimate both the genetic (ρ_g) and the environmental (ρ_e) correlations between traits. The phenotypic correlation (ρ_p) is derived from these two constituent correlations and the heritabilities of the traits:

$$\rho_p = \sqrt{(h^2_1 h^2_2)}\, \rho_g + \sqrt{(1- h^2_1)}\, \sqrt{(1- h^2_2)}\, \rho_e.$$

Examination of the underlying determinants of phenotypic correlations provides information on the role of pleiotropic genetic effects (i.e., common genetic factors that affect several phenotypes). Additionally, the partitioning of phenotypic correlations into genetic and environmental components is potentially valuable since hidden relationships between traits can be revealed (10).

Estimates of the phenotypic, genetic, and environmental correlations between thrombosis and the quantitative phenotypes were obtained using maximum-likelihood estimation. All hypotheses were tested using likelihood-ratio test statistics (8, 9). Because 12 of the 21 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 (11, 12).

RESULTS

Covariate effects and heritabilities

Table 1 shows the regression coefficients for the environmental covariates, estimated along with the effects of heredity and household. There was a significant effect of age on all of the

phenotypes except Factor XIII subunits. In addition, there was a significant effect of sex on α_2-antiplasmin, Factor XIII-A subunit, sP-selectin, vitamin B 12 and serum folate. The use of oral contraceptives influenced the Tp-TM T related phenotypes and smoking significantly decreased serum folate levels and increased the levels of Factor XIII subunits.

Table 2 presents the estimated components of phenotypic variance. Components of variance are shown for the most parsimonious model (i.e., the model that best fits the observed data and exhibits the minimum complexity) for each phenotype, including only the significant sources of variation. The remaining variance not accounted for in Table 2 is attributable to individual-specific random environmental influences and random error. All of these traits had a significant genetic component (heritability), ranging from 22% for R2 to 78% for β_2-glycoprotein I. The proportion of the residual phenotypic variability accounted for by shared household effects was significant only for serum folate, whole blood folate, vitamin B12, R1 and R2.

Correlations between thrombosis liability and quantitative phenotypes

Table 3 shows the results of bivariate genetic analyses of thrombosis with each of the quantitative physiological traits. Of these, only the 3 traits related with Tp-TM T exhibited significant phenotypic correlation with thrombosis susceptibility. Serum folate was genetically correlated with liability to thrombosis ($\rho_G = -0.579$, $p = 0.015$). The phenotypes related to Tp-TM T also demonstrated significant genetic correlations with thrombosis: R1 had $\rho_G = -1.000$, p= 0.00006; R2, $\rho_G = -0.974$, $p = 0.032$; and R1/R2, $\rho_G = -0.834$, $p = 0.000004$. Significant environmental correlations were observed only for serum folate ($\rho_E = 0.598$, p= 0.003) and for R1/R2 ($\rho_E = 0.601$, p= 0.005). Other phenotypes like α_2-antiplasmin ($\rho_E = 0.389$, p=0.08) and R1 ($\rho_E = 0.380$, p= 0.063) almost reached statistical significance for environmental correlation.

These significant genetic correlations provide strong evidence for pleiotropic genes contributing to the covariation between serum folate, the traits related with Tp-TM T and thrombotic risk. The results shown in Table 3 demonstrate how low phenotypic correlations may misrepresent the true underlying relationships. For example, serum folate failed to show a significant phenotypic correlation with thrombosis ($\rho_P = -0.018$, not significant); however, we obtained strong evidence for correlations between genetic ($\rho_G = -0.579$, $p = 0.015$) and environmental ($\rho_E = 0.598$, p= 0.003) effects with thrombosis. The magnitudes of the genetic and environmental correlations between serum folate and thrombosis are similar but exhibit different signs. When such differences in sign appear, the phenotypic correlation is attenuated or can even disappear (as in this example), although the underlying components suggest much stronger correlations.

DISCUSSION

In a previous paper, we reported that several plasma hemostasis phenotypes were influenced by genetic factors (7). The present report extends these studies and clearly demonstrates that genetic factors influence the variability of additional hemostasis traits. In fact, we found that heredity was the largest identifiable determinant of the quantitative variation in these traits. Shared environment had a substantial effect on only a few phenotypes and was most apparent for folate-related traits, vitamin B12 and two of the Tp-TM T phenotypes.

Our estimates of heritabilities are conservative because we considered the influence of additive genetic effects only. If other non-additive sources of genetic variance exist, such, as dominance or epistasis, then our heritabilities would be underestimated. Heritability can also be decreased by measurement error. One way to increase the genetic signal-to-noise ratio would be to eliminate measurement error. In general, the measures considered have modest errors with inter- and intra-assay coefficients of variation lesser than 10%. The error for Factor XIII A subunit is larger (12.1%) and may have contributed to its relatively low heritability. However, measurement error of this magnitude is likely to have only a small effect on the heritability estimates. If error were eliminated via multiple measures, the estimated heritability would increase only slightly (around 2-

3%). However, the complete elimination of measurement error is not feasible in large studies, such as ours.

In this study, we have statistically controlled for the effects of demographic and exogenous covariates such as smoking. We have consciously avoided employing biological covariates that may themselves be influenced by genes. For example, composite phenotypes such as Tp-TM T – related traits could be influenced by a number of intermediate traits such as factor VII or prothrombin plasma levels. If we were to correct the Tp-TM T phenotypes 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.

There have been very few studies of the heritability of these hemostasis phenotypes. It is difficult to compare our results with those of other studies because most of them have not used family-based designs appropriate for assessing genetic effects. However, the study of soluble P-selectin in Mexican-American families in the San Antonio Family Heart Study (13) is directly comparable to our study. Blangero *et al* estimated sP-selectin heritability to be between 60-70%, which is probably not different from our estimate of 42% for this phenotype in Spanish families, given the estimated standard errors. Ariëns *et al* reported the heritabilities for Factor XIII subunits in a twin study: They attributed 38% of the plasma variation of Factor XIII S and 61% of the variation of Factor XIII A to genetic effects (14). Although not directly related with the present phenotypes, Mitchell *et al* published a heritability of 46% for a folate-related trait, red blood cell folate which is obtained by correcting whole blood folate by hematocrit (15). Overall, there appears to be consistency between these estimates based on twin studies and our own. However, our study was the only one that was designed explicitly to disentangle genetic effects from shared environmental effects. To our knowledge, there are no reports on the heritabilities of the other 8 phenotypes that we studied in this paper.

Although statistically significant, the heritability and household effects observed for whole blood folate must be interpreted cautiously because the sample size was relatively small for an accurate estimation of the variance components (Table 2). This sample size was considered too small for the bivariate analysis used in the estimation of the correlations (Table 3) which were not performed for this phenotype.

The exploitation of data from extended pedigrees, methodically ascertained to allow general population inferences, like those in our GAIT Project, can be very useful in the search for genetic factors influencing common thrombosis. Currently, most of the knowledge regarding the genetic factors implicated in thrombosis comes from association studies that employ case-control designs to explore known polymorphic variations in candidate genes (16-19). Using extended pedigrees, we have recently found that APC resistance, factors VII, VIII, IX, XI and XII, t-PA, von Willebrand factor and homocysteine are genetically correlated with thrombotic risk (6). These results indicate that genes (most of them probably unknown) jointly influence both disease risk and physiological variation in risk factors. The present report adds four phenotypes (serum folate and the 3 Tp-TM T phenotypes) to those described previously. It is interesting to note that serum folate level is one of the factors that influence homocysteine metabolism (20). Normally, it is considered a dietary factor, depending upon environment more than heredity. However, our results strongly indicate that heredity plays a more significant role ($h^2 = 0.32$) than household effects, including dietary factors ($c^2 = 0.15$). The strong genetic correlation that we found between serum folate and thrombotic risk is provocative because it suggests an additional mechanism for hyperhomocysteinemia-associated thrombosis, whose physiopathology is not well understood (21). It is noteworthy that we found a relatively high genetic correlation between thrombotic risk and vitamin B12 levels ($\rho_G = -0.347$, $p = 0.14$). Although this result was not statistically significant, it may be productive to re-examine this trend in a larger sample.

The almost complete statistical correlation that we found between our modified thromboplastin time in presence or absence of thrombomodulin (Tp-TM T phenotypes) and thrombotic risk is impressive. The estimated genetic correlation of –1.000 between R1 and thrombotic risk may mean that *exactly* the same genes influence R1 and the risk of thrombosis. At the very least it suggests that the genetic influences on R1 and on thrombotic risk overlap extensively and/or that the genetic influences on one may be a subset of the genetic influences on the other. Our method for determining Tp-TM T traits can be considered as an indirect way to

measure the thrombin generation and the procoagulant potential of plasma. Therefore, their relationship with the risk of thrombosis is not surprising. Interestingly, we have previously found high heritabilities for some other phenotypes related with coagulation times such as APTT (83%), prothrombin time (50%) and APC resistance (71%) (7). Nevertheless, only those coagulation times that were determined using special reagents with anticoagulant function like activated protein C and thrombomodulin showed a significant genetic correlation with thrombotic risk. The ρ_G between APC resistance and thrombotic risk was estimated as −0.650 (6). If we are successful in our search for the genes that determine variability in these traits, then these genes would be new candidates for the explanation of idiopathic thromboembolic disease.

A primary goal of modern genetic analysis is to partition the genetic variability in a phenotype into components attributable to specific quantitative trait loci (QTLs). Such goals can now be attained using powerful new methods of quantitative trait linkage analysis on human pedigree data such as that collected for our GAIT study. These new methods for detecting linkage will also provide estimates of chromosomal location and, equally important, unbiased estimates of the relative importance of specific QTLs for the general population. These estimates will be essential for the evaluation 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 influencing risk of thrombosis. This information may then be used for predicting individual-specific risk early enough in life to consider prophylactic intervention.

ACKNOWLEDGEMENTS

This study was supported by grants RED 98/14 from Generalitat de Catalunya, DGICYT Sab 94/0170 from the Ministerio de Educacion y Ciencia, FIS 97/2032 from the Ministerio de Sanidad y Consumo, Spain and NIH grant MH59490.

We are grateful to the doctors who assisted in recruitment of thrombophilic pedigrees: Dr Javier Rodríguez Martorell (Hospital Universitario Puerta del Mar, Cádiz), Dr Carmen Araguás (Hospital Arnau de Vilanova, Lleida), Dr Francisco Velasco (Hospital Reina Sofía, Córdoba), Dr Montserrat Maicas (Hospital General de Albacete) and Dr Dilia Brito (Hospital Carlos Haya, Málaga). Finally, we are deeply grateful to the families who participated in this study.

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Table 1. Regression coefficients for statistically significant covariate effects

	Mean (males)	Female sex	Age:M*	Age:F*	Age^2:M*	Age^2:F*	Smoking	Use of oral Contracept.
R1 (no units)	1.09		–0.0018 ‡		0.0014 ¶			–0.220 §
R2 (no units)	1.05		–0.0022 ¶		0.0001 ¶	–0.00008 ‡		–0.073 †
R1/R2 (no units)	1.03			0.0012 †	0.00004 ‡			–0.135 •
α_2-antiplasmin (%)	88.81	4.21‡	–0.073 †					
β_2-glycoprotein I (%)	109.31		0.179 •					
Factor XIII A (%)	95.19	–6.08 †					8.80 ‡	
Factor XIII S (%)	102.34						5.43 †	
Prekallikrein (%)	106.57		0.327 ¶		–0.009 •			
P-selectin (ng/mL)	52.55	–8.23 •		0.256 ‡				
Vitamin B12(pmol/L)	353.02	92.41 ¶	–1.32 ‡	1.794 ‡	0.105 ¶	–0.101 §		
Serum folate (nmol/L)	15.61	1.52 ‡			0.003 ‡	–0.003 ‡	–2.84 ¶	
Whole blood folate (nmol/L)	249.99		1.02 ‡			–0.021 †		

*The coefficients represent the effect of change at 10-year intervals. M, male; F, female.
R1 is the ratio between the thromboplastin time of the patient's plasma versus the thromboplastin time of the control plasma, in the presence of thrombomodulin. R2 is the ratio between those thromboplastin times in the absence of thrombomodulin. R1/R2 is the ratio between R1 and R2.
Levels of significance: † $p< 0.10$, ‡ $p< 0.05$, § $p< 0.01$, • $p< 0.001$, ¶ $p<0.0001$.

Table 2. Components of variance from the most parsimonious model with standard errors

Phenotype	Heritability	SE	Household	SE
R1	0.41 •	0.11	0.16 ‡	0.07
R2	0.22 ‡	0.11	0.12 †	0.08
R1/R2	0.56 ¶	0.08	–	
α_2-antiplasmin	0.32 •	0.09	–	
β_2-glycoprotein I	0.78 ¶	0.10	–	
Factor XIII A	0.32 §	0.09	–	
Factor XIII S	0.48 ¶	0.09	–	
Prekallikrein	0.48 ¶	0.09	–	
sP-selectin	0.43 •	0.11	–	
Vitamin B12	0.35 •	0.09	0.13 †	0.07
Serum folate	0.32 §	0.09	0.15 ‡	0.07
Whole blood folate	0.34 ‡	0.17	0.39 •	0.10
Liability to Thrombosis*	0.61 §	0.16	–	

(–) Not statistically different from zero.
(*) From Souto *et al* (6)
R1, R2 and R1/R2: see Table 1
Significance levels: † $p<0.05$, ‡ $p<0.01$, § $p< 0.0001$, • $p<0.00001$, ¶ $p<0.0000001$

Table 3. Phenotypic, genetic and environmental correlations of the phenotypes with liability to thrombosis.

Phenotype	ρ_P	p-value	ρ_G	p-value	ρ_E	p-value
R1	−0.312	0.0006	−1.000	0.00006	0.380	0.063
R2	−0.200	0.031	−0.974	0.032	0.070	NS
R1/R2	−0.226	0.015	−0.834	0.000004	0.601	0.005
α_2-antiplasmin	0.125	NS	−0.165	NS	0.389	0.08
β_2-glycoprotein I	0.100	NS	0.119	NS	0.060	NS
Factor XIII A	0.057	NS	0.273	NS	−0.128	NS
Factor XIII S	0.095	NS	0.208	NS	−0.036	NS
sP-selectin	0.084	NS	0.199	NS	−0.031	NS
Prekallikrein	0.136	NS	0.007	NS	0.295	NS
Serum folate	−0.018	NS	−0.579	0.015	0.598	0.003
Vitamin B12	−0.065	NS	−0.347	0.14	0.357	NS
Whole blood folate*	Not done		Not done		Not done	

ρ_P = phenotypic correlation, ρ_G = genetic correlation, ρ_E = environmental correlation.
NS denotes non-significant p-values > 0.15
R1, R2 and R1/R2: see Table 1.
* Due to the small sample size, there was insufficient statistical power for estimation of correlations with whole blood folate.

6. RESUMEN DE RESULTADOS Y DISCUSIÓN

6.1 HEREDABILIDAD DE LOS FENOTIPOS DE LA HEMOSTASIA

En la Tabla 5 se muestran las estimaciones de heredabilidad obtenidas para 40 fenotipos complejos relacionados con la coagulación, la fibrinolisis y potencialmente con la enfermedad tromboembólica. Estas estimaciones se han obtenido una vez controlado el efecto de las covariables ambientales recogidas en el estudio (edad, sexo, tabaquismo y uso de anticonceptivos orales). Gracias a la corrección de reclutamiento, los resultados son aplicables a la población general española, origen de las familias incluidas. La descomposición de la variancia que refleja la Tabla 5 es el resultado del modelo más parsimonioso (es decir, el modelo que explica mejor los datos observados y que posee la complejidad mínima) para cada fenotipo. La variabilidad restante hasta completar el 100%, no mostrada en la Tabla, es debida a las influencias ambientales aleatorias específicas de cada individuo.

Todos los fenotipos, excepto el dímero D, muestran un componente genético significativo. La mayoría oscilan entre el 22% y el 55% de la variabilidad residual del fenotipo (aquella que permanece tras controlar las covariables ambientales conocidas). El TTPA, la β_2-glycoprotein I, la RPCa y el factor XII presentan influencias genéticas excepcionalmente grandes, del 83%, 78%, 71% y 67% respectivamente. En contraste, el dímero D no parece tener un componente hereditario, puesto que la estimación de heredabilidad del 10.9% no es estadísticamente distinta de 0 ($p = 0.07$).

La proporción de variabilidad residual fenotípica debida a efectos domiciliarios compartidos tiende a ser mucho menor que la debida a los genes. Tan sólo es significativa en 13 fenotipos y su valor oscila entre 9.5% para la proteína S funcional y 39% para el folato total.

Las estimaciones del componente genético de estos fenotipos son conservadoras, porque el modelo matemático usado sólo contempla los efectos aditivos de los genes. En el caso, muy probable, que existan otras fuentes de variancia genética no aditivas, tales como la dominancia o la interacción - epistasis - entre genes, entonces los valores reales de heredabilidad serían aún mayores.

El proyecto GAIT es el primero en realizar un estudio sistemático de heredabilidades de la Hemostasia. Con anterioridad a 1995 habían aparecido algunos estudios muy esporádicos, realizados con diseños de menor poder estadístico y menos

adecuados para distinguir entre los efectos del ambiente y los genéticos sobre fenotipos complejos. Prácticamente todos estaban basados en estudios de gemelos. Los fenotipos cuyas heredabilidades se habían reportado eran factor von Willebrand, factor VIII y factor IX *(Orstavik y col 1985)*, fibrinógeno *(Humphries y col 1987, Hamsten y col 1987)*, homocisteína *(Reed y col 1991, Berg y col 1992)* y HRG *(Boomsma y col 1993)*.

Contemporáneamente al proyecto GAIT se han publicado otros estudios aislados sobre heredabilidades de otros fenotipos como PAI-1 *(Hong y col 1997, Pankow y col 1998)*, factor VII *(Hong y col 1999)*, P-selectina *(Blangero y col 1999)*, subunidades del factor XIII y t-PA *(Ariëns y col 1999)*, RPCa, proteína C y antitrombina *(Tosetto y col 2000)*. La comparación entre los resultados del proyecto GAIT y los de otros estudios es difícil porque la mayoría no han usado diseños basados en familias, apropiados para valorar los efectos genéticos. En general se observa una cierta consistencia entre las estimaciones, teniendo en cuenta, por ejemplo, que los estudios en gemelos siempre tienden a sobrevalorar la heredabilidad, puesto que son incapaces de distinguir las fuentes de variación provocadas por los genes comunes de aquellas debidas a compartir un mismo ambiente.

Tabla 5. Componentes de la variancia (heredabilidad y efecto domiciliario) con su error estándar (EE), en orden decreciente de heredabilidades

Fenotipo	Heredabilidad	EE	Efecto domiciliario	EE
TTPA	0.830 §	0.07	–	
β_2-glycoprotein I	0.780 §	0.10	–	
RPCa	0.713 §	0.08	–	
Factor XII	0.673 §	0.08	–	
R1/R2	0.560 §	0.08	–	
Factor VII	0.523 §	0.09	–	
HRG	0.522 §	0.09	–	
TFPI	0.516 §	0.09	–	
Tº Protrombina	0.504 §	0.08	–	
Proteína C	0.501 §	0.09	–	
Factor II	0.492 §	0.09	–	
Antitrombina	0.486 §	0.09	–	
Factor XIII S	0.480 §	0.09	–	
Precalicreína	0.480 §	0.09	–	
Prot S libre	0.460 §	0.09	0.108 *	0.06
Prot S funcional	0.453 §	0.10	0.095 *	0.06
Factor XI	0.452 §	0.10	0.162 *	0.08
Factor V	0.442 §	0.09	0.133 *	0.07
Cofactor II Heparina	0.439 §	0.09	–	
Factor X	0.434 §	0.13	0.135 *	0.08
P-selectina soluble	0.430 §	0.11	–	
R1	0.410 §	0.11	0.160 ‡	0.07
Factor VIII	0.400 §	0.09	–	
Factor IX	0.387 §	0.09	–	
Vitamina B12	0.350 §	0.09	0.130 †	0.07
Folato total sangre	0.340 ‡	0.17	0.390 §	0.10

Tabla 5 (continuación)

Fenotipo	Heredabilidad	EE	Efecto domiciliario	EE
Fibrinógeno	0.336 §	0.10	0.137 *	0.06
α_2-antiplasmina	0.320 §	0.09	–	
Factor XIII A	0.320 §	0.09	–	
Folato sérico	0.320 §	0.09	0.150 ‡	0.07
Factor Willebrand	0.318 §	0.11	–	
PAI-1	0.298 §	0.08	0.139 †	0.06
t-PA	0.268 §	0.07	–	
Homocisteína	0.244 §	0.08	–	
Plasminógeno	0.236 †	0.10	–	
Prot S total	0.223 †	0.11	0.212 ‡	0.06
R2	0.220 ‡	0.11	0.120 †	0.08
Factor tisular	0.167 †	0.08	–	
Dímero D	0.109 #	0.09	–	
Riesgo de trombosis	0.610 §	0.16	–	

#p < 0.10, *p < 0.05, †p < 0.01, ‡p < 0.001, §p < 0.0001

(–) No diferente estadísticamente de 0.

R1 es la *ratio* entre el tiempo de protrombina del plasma del paciente y el mismo tiempo del plasma control, en presencia de trombomodulina. R2 es la *ratio* entre los mismos tiempos de protrombina pero sin trombomodulina. R1/R2 es la *ratio* entre R1 y R2

TTPA: Tiempo de tromboplastina parcial activado
RPCa: Resistencia a la proteína C activada
HRG: Glicoproteína rica en histidina
TFPI: Inhibidor de la vía del factor tisular
PAI-1: Inhibidor del activador del plasminógeno tipo 1
t-PA: Activador tisular del plasminógeno

6.2 HEREDABILIDAD DEL FENOTIPO "RIESGO DE TROMBOSIS"

Este es uno de los resultados inéditos más importantes de la presente tesis. El fenotipo "riesgo de trombosis" no es observable ni mensurable directamente, pero se puede evaluar gracias a un método de Máxima Verosimilitud aplicado en familias y que modeliza el estado de cada sujeto (sano *versus* enfermo) como un proceso continuo con un umbral que separa los afectos de trombosis de los no afectos *(Duggirala y col 1997, Williams y col 1999b)*. La construcción de este fenotipo teórico se realiza a partir de los eventos tromboembólicos observados en la muestra de las 21 familias que se resumen en la Tabla 6. Un total de 53 individuos, entre los 398 del estudio, presentaron algún evento de trombosis venosa o arterial. La mayoría (47) pertenecen a las familias reclutadas por trombofilia, pero también se observaron 6 individuos afectos en las familias reclutadas al azar. Otro dato relevante es que en ocho de las familias se observaron a la vez casos de los dos tipos clásicos de trombosis, venosa o arterial. Cuando se consideran por separado los tipos de trombosis, se distinguen 40 individuos con alguna trombosis venosa (edad promedio del primer episodio 39.7 años) y 17 individuos con una o más trombosis arteriales (edad promedio del primer episodio a los 61 años). Existen, por lo tanto, 4 sujetos con antecedentes de trombosis venosa y arterial.

Tabla 6. Número y porcentaje de individuos examinados con algún episodio de trombosis según categorías clínicas y edad media (años) del primer diagnóstico.

Diagnóstico	N	%	Edad media diagnóstico
TROMBOSIS VENOSA			
TVP	28	52.8	40.3
Embolia pulmonar	9	17.0	45.6
Tromboflebitis superficial	14	26.4	41.2
Otras trombosis venosas	3	5.7	58.0
Cualquier trombosis venosa	40	75.5	39.7
TROMBOSIS ARTERIAL			
Infarto de miocardio	4	7.5	66.5
Angina de pecho	4	7.5	57.3
AVC isquémico establecido	6	11.3	61.0
AVC tipo transitorio	5	9.4	55.4
Cualquier trombosis arterial	17	32.1	61.0
CUALQUIER TROMBOSIS	53	100.0	44.5

Nuestros resultados evidencian de forma contundente una gran influencia genética en el riesgo de trombosis. La heredabilidad del riesgo de trombosis (considerada la trombosis en sentido amplio, arterial + venoso) es del 61 ± 16% (Tabla 5). Esto indica que tras corregir los efectos de la edad y sexo (covariables incluidas en el modelo umbral) el 61% de la variación en el riesgo de trombosis en la población general debe atribuirse a factores genéticos. No se detectan efectos domiciliarios compartidos sobre el riesgo de trombosis, por lo que con mucha probabilidad la dieta no influye sobre la enfermedad tromboembólica o lo hace de un modo irrelevante.

Se han obtenido dos resultados adicionales relacionados con la heredabiliad del riesgo trombótico de gran interés:

1. Si se considera sólo el fenotipo “trombosis venosa” y se construye la curva del riesgo de trombosis venosa (a partir de los 40 individuos con este diagnóstico) la heredabilidad que se obtiene no es significativamente distinta de la obtenida para la trombosis en sentido amplio.
2. Si consideramos a la “trombosis venosa” y a la “trombosis arterial” como dos rasgos distintos, construimos las respectivas curvas de riesgo y estudiamos sus correlaciones, (ver apartado 2.4) obtenemos:

   Correlación fenotípica 0.333 (p=0.0126)

   Correlación genética 0.550 (p=0.09)

   Además, la correlación genética no resulta ser significativamente distinta de 1

La interpretación combinada de estos datos sugiere que los genes subyacentes al riesgo de trombosis venosa y los subyacentes al riesgo de trombosis arterial son, en gran parte, los mismos.

En comparación con otras enfermedades complejas, investigadas mediante los mismos métodos usados en este estudio, la heredabilidad del 61% estimada para la trombosis es de las más altas. Como ejemplos, podemos citar el 48% para la litiasis vesicular *(Duggirala y col 1999b),* el 53% para la diabetes tipo II *(Duggirala y col 1999a)*, entre el 49-60% para el alcoholismo *(Williams y col 1999a)* o el 63% para la masa grasa, como medida directa de la obesidad *(Comuzzie y col 1997)*.

6.3 ESTUDIO DE LAS CORRELACIONES ENTRE LOS FENOTIPOS DE LA HEMOSTASIA

Como ya se ha indicado en la Introducción, los métodos de estadística genética basados en el análisis de los componentes de la variancia permiten estudiar de una manera más profunda las correlaciones entre fenotipos complejos. Son capaces de discriminar las fuentes de correlación debidas al efecto de los genes de las debidas a causas ambientales.

Se han estudiado todas las posibles parejas (n = 780) de fenotipos que se derivan de los 40 fenotipos definidos en el proyecto (ver lista en Tabla 5). Desde el punto de vista de los objetivos finales del proyecto GAIT (localización de genes que influyen sobre los fenotipos de la Hemostasia y sobre el riesgo de trombosis) adquieren una importancia relevante las parejas de fenotipos que presentan correlación *genética* significativa. Esto se debe a que en los modelos matématicos usados en el Análisis de Ligamiento se puede conseguir mayor poder estadístico y por tanto mayor probabilidad de localizar un QTL si este influye simultáneamente a dos fenotipos (pleiotropía). Aproximadamente unas 100 de las 780 parejas posibles han resultado estar correlacionadas genéticamente. Muchos de estos datos aún no han sido publicados.

Se presentan en esta Tesis dos grupos de correlaciones de gran interés:

6.3.1 Correlaciones entre los fenotipos dependientes de la vitamina K

Las proteínas plasmáticas de la Hemostasia que precisan la vitamina K para su síntesis correcta son los factores II, VII, IX y X, la proteína C y la proteína S. Estas proteínas poseen una gran homología estructural y en la secuencia de aminoácidos *(Furie y Furie 1992)*. Además, todas ellas parecen haber evolucionado de una proteína ancestral común, a través de procesos de duplicación y divergencia genética *(Patthy 1990)*. Si consideramos esta relación evolutiva entre los actuales genes estructurales que codifican las proteínas dependientes de la vitamina K, es probable que tanto los genes implicados como las proteínas que codifican tengan mecanismos reguladores comunes. Una manera de aportar luz en relación con esta hipótesis ha sido estudiar todas las correlaciones genéticas entre los fenotipos relacionados. En la Tabla 7 se observa como 18 de las 28 parejas presentan correlación genética claramente significativa y como 6 parejas más muestran la misma tendencia ($p<0.10$).

Las correlaciones ambientales son significativas en 25 parejas de fenotipos (ver Tabla 7).

El análisis de las posibles correlaciones debidas al efecto domiciliario sólo tiene sentido entre aquellos fenotipos que demostraron tener un efecto domiciliario (ver Tabla 5). Estos fueron el factor X y los fenotipos relacionados con la proteína S (total, libre y funcional). Se observa una ausencia de correlación por efectos domiciliarios entre el factor X y la proteína S (Tabla 7).

¿Cuál es la interpretación conjunta de los resultados mostrados?. La explicación biológica más sencilla implica la presencia de genes reguladores comunes, con efectos pleiotrópicos sobre las proteínas plasmáticas dependientes de la vitamina K. Por otro lado, el alto grado de correlación ambiental que presentan estas proteínas indica que los factores ambientales que las influyen son, muy probablemente, en gran parte los mismos. Sin embargo, la ausencia de componente domiciliario en la mayoría de estos fenotipos (II, VII, IX y proteína C) así como la ausencia de correlación domiciliaria entre el factor X y la proteína S, hace muy improbable que la dieta sea uno de los factores ambientales responsable de las correlaciones entre los fenotipos dependientes de la vitamina K.

Tabla 7

Correlaciones fenotípicas, genéticas, ambientales y domiciliarias entre todos los pares de fenotipos dependientes de la vitamina K.

	Correlaciones			
Fenotipos	Fenotípica	Genética	Domiciliaria	Ambiental
FII – FVII	0.39[1]	0.23[5]	-	0.55[1]
FII – FIX	0.46[1]	0.53[3]	-	0.41[2]
FII – FX	0.54[1]	0.35[4]	-	0.82[1]
FII – Prot. C	0.45[1]	0.31[4]	-	0.59[1]
FII – PS libre	0.29[1]	0.46[3]	-	0.30[3]
FII – PS total	0.41[1]	0.86[1]	-	0.19[6]
FII – PS func.	0.23[1]	0.26[6]	-	0.33[3]
FVII – FIX	0.35[1]	0.08[6]	-	0.55[1]
FVII – FX	0.48[1]	0.14[5]	-	0.71[1]
FVII – Prot. C	0.46[1]	0.42[3]	-	0.51[1]
FVII – PS libre	0.20[2]	0.22[5]	-	0.24[4]
FVII – PS total	0.29[1]	0.57[4]	-	0.21[5]
FVII – PS func.	0.23[1]	0.36[4]	-	- 0.02[6]
FIX – FX	0.43[1]	0.23[5]	-	0.53[1]
FIX – Prot. C	0.42[1]	0.33[4]	-	0.48[1]
FIX – PS libre	0.30[1]	0.35[4]	-	0.23[4]
FIX – PS total	0.29[1]	0.51[5]	-	0.30[3]
FIX – PS func.	0.19[1]	-0.13[6]	-	0.35[3]
FX – Prot. C	0.41[1]	0.15[5]	-	0.61[1]
FX – PS libre	0.45[1]	0.65[2]	-0.11[6]	0.37[3]
FX – PS total	0.45[1]	0.86[3]	-0.23[6]	0.30[4]
FX – PS func.	0.42[1]	0.65[3]	0.12[6]	0.28[4]
Prot. C – PS libre	0.31[1]	0.33[3]	-	0.33[3]
Prot. C – PS total	0.35[1]	0.67[3]	-	0.25[4]
Prot. C – PS func.	0.28[1]	0.12[6]	-	0.37[3]
PS libre – PS total	0.63[1]	0.80[3]	0.80[1]	0.55[1]
PS libre– PS func.	0.62[1]	0.65[3]	0.61[4]	0.58[1]
PS total – PS func.	0.42[1]	0.76[3]	0.12[6]	0.29[4]

(1) p<0.0001, (2) p<0.001, (3) p<0.01, (4) p< 0.05, (5) p<0.10, (6) no significativo

6.3.2 Fenotipos correlacionados genéticamente con el riesgo de trombosis

Se ha estudiado la correlación de cada uno de los fenotipos plasmáticos con el fenotipo "riesgo de trombosis". En la Tabla 8 se muestran los 13 fenotipos que han presentado alguna correlación significativa (fenotípica, genética o ambiental). Todos ellos tienen correlación genética con el riesgo de trombosis. Por definición, esto implica que algunos de los genes que regulan a estos fenotipos plasmáticos, son también responsables de la enfermedad tromboembólica. El resto de los fenotipos no han presentado correlación con el riesgo de trombosis, pero no se puede descartar que alguno de ellos la tenga. En tal caso, serían necesarios estudios más potentes, por ejemplo con mayor número de familias y de mayor tamaño, para alcanzar resultados significativos.

Los resultados son coherentes con varios estudios epidemiológicos previos de asociación (caso/control) que habían relacionado a algunos estos fenotipos con el riesgo de trombosis. Los niveles de factor VIII y de factor von Willebrand se han asociado a trombosis venosa *(Koster y col 1995)* y a trombosis arterial *(Folsom y col 1997)*. El aumento de los niveles de homocisteína en plasma se relaciona con mayor riesgo de trombosis venosa *(den Heijer y col 1996)* y también de trombosis arterial en forma de infarto de miocardio *(Nygard y col 1997).* También la RPCa presenta una relación inversa con el riesgo de trombosis venosa *(de Visser y col 1999).* Algunos autores han hallado relación entre los niveles de factor XII *(Kohler y col 1998)* y de t-PA *(Ridker y col 1993, Carter y col 1998)* y la enfermedad trombótica arterial. Muy recientemente, han aparecido resultados implicando a los niveles elevados de factor IX *(Vlieg y col 2000)* y de factor XI *(Meijers y col 2000)* en el riesgo de trombosis venosa. Existen estudios sobre el factor VII y el riesgo de infarto de miocardio con resultados discrepantes ya que en alguno se observa una asociación positiva *(Iacoviello y col 1998)* mientras que en otros no se detecta relación *(Doggen y col 1998)*.

Los resultados del proyecto GAIT vienen a confirmar las sospechas despertadas por estos estudios. Cabe recordar que el diseño común a todos ellos, basados en comparaciones entre cohortes de pacientes y controles sanos, no emparentados entre sí, hace imposible establecer una relación de causa/efecto entre el factor de riesgo y la enfermedad. Además los estudios de asociación que involucran factores genéticos son siempre muy propensos a establecer resultados positivos falsos. Sin embargo, la técnica

estadística que utiliza el proyecto GAIT, aparte de ser mucho más potente, es muy poco probable que obtenga falsos positivos (se ha realizado sobre una población homogénea, sin estratos genéticos ocultos puesto que se trata de familias). El establecimiento de correlaciones genéticas entre la variabilidad de estos fenotipos y la variabilidad del riesgo de trombosis, permite formular con gran contundencia la implicación causal de estos fenotipos en el desarrollo de la enfermedad.

Hemos detectado además 2 nuevos fenotipos relacionados estrechamente con el riesgo de trombosis. Uno de ellos es un tiempo de coagulación, el tiempo de protrombina en presencia de trombomodulina. Presenta una correlación genética impresionante con el riesgo de trombosis, de carácter negativo y muy próxima a la correlación total ($\rho_G = -1$). Esto puede significar que exactamente los mismos genes que influyen este tiempo de coagulación influyen el riesgo de trombosis. Como mínimo, sugiere que las influencias genéticas son en gran parte las mismas o bien que los genes que influyen en el tiempo de protrombina modificado son un subconjunto de los genes que influyen en el riesgo de trombosis.

El segundo fenotipo nuevo es el folato sérico. Se observa una fuerte correlación genética negativa ($\rho_G = -0.58$) con el riesgo de trombosis. Por otra parte, también presenta una correlación ambiental intensa, pero positiva ($\rho_E = 0.60$). Como consecuencia, ambas fuentes de correlación representan fuerzas opuestas que se manifiestan como una ausencia de correlación fenotípica ($\rho_F = -0.02$, no significativa). Este ejemplo ilustra otra de las grandes virtudes del método utilizado: allí dónde aparentemente no hay correlación o es débil, pueden subyacer intensas correlaciones de carácter opuesto, imposibles de revelar con los métodos estadísticos tradicionales. Observando la Tabla 8 también se comprueba este fenómeno en relación con el factor VII. Y otros fenotipos con correlaciones fenotípicas poco llamativas, tienen en realidad una muy intensa correlación genética con el riego de trombosis (RPCa, factor VIII, factor IX, factor XI, homocisteína, t-PA y factor von Willebrand).

Estos resultados se cuentan entre los más relevantes de la tesis por su trascendencia clínica. A partir de ahora, el objetivo de la investigación deberá ser la búsqueda de los QTL subyacentes a estos fenotipos, que se han convertido en fenotipos intermediarios del riesgo de enfermedad. Con gran probabilidad, en estos QTL se hallarán parte de los genes responsables de la trombosis en la población general.

Tabla 8

Correlaciones fenotípicas, genéticas y ambientales de los fenotipos de la Hemostasia con el fenotipo "riesgo de trombosis". Sólo se muestran fenotipos con alguna correlación significativa (valor p < 0.05)

Fenotipo	ρ_F	valor p	ρ_G	valor p	ρ_E	valor p
RPCa	–0.230	0.0003	**–0.650**	1×10^{-6}	0.669	0.0006
FVII	0.025	N.S.	**–0.354**	0.0564	0.568	0.0091
FVIII	0.288	0.0002	**0.689**	0.0005	–0.126	N.S.
FIX	0.151	0.0787	**0.597**	0.0131	–0.198	N.S.
FXI	0.209	0.0180	**0.564**	0.0245	0.070	N.S.
FXII	0.172	0.0339	**0.351**	0.0500	–0.145	N.S.
Homocisteína	0.227	0.0018	**0.652**	0.0015	–0.028	N.S.
t-PA	0.180	0.0002	**0.752**	0.0070	–0.099	N.S.
F vW	0.261	0.0010	**0.729**	0.0005	–0.181	N.S.
R1	–0.312	0.0006	**–1.000**	0.00006	0.380	0.063
R2	–0.200	0.0310	**–0.974**	0.0320	0.070	N.S.
R1/R2	–0.226	0.0150	**–0.834**	0.000004	0.601	0.005
Folato sérico	–0.018	N.S.	**–0.579**	0.0150	0.598	0.003

ρ_P = correlación fenotípica, ρ_G = correlación genética, ρ_E = correlación ambiental

N.S. denota valor p no significativo > 0.10

FvW representa al factor von Willebrand. Para el resto de abreviaturas, ver Tabla 5.

6.4 ESTUDIO DE LA MUTACIÓN G20210A EN EL GEN DE LA PROTROMBINA

En 1996 se describió un dimorfismo en la posición 20210 del gen de la protrombina consistente en un cambio del nucleótido guanina (G) por adenina (A) y que se asociaba con un riesgo de trombosis unas 3 veces mayor en los portadores (alelo A) que en los normales (alelo G). Además los portadores presentaban unos niveles plasmáticos de factor II más elevados que los no portadores *(Poort y col 1996)*.

El hecho de que la posición 20210 corresponde a una zona no codificante del gen (situada en el extremo 3' del gen) obligaba a tener cautela antes de aceptar este dimorfismo como un factor de riesgo. Más aún teniendo en cuenta que se podía tratar de un falso positivo, o que podía no ser realmente el factor causal, sino encontrarse en desequilibrio de ligamiento con el verdadero factor genético. Sin embargo, múltiples estudios de asociación posteriores confirmaron la asociación de la mutación PT G20210A con la trombosis venosa y con los niveles de factor II.

El estudio GAIT ha permitido establecer inequívocamente algunos hechos relacionados con este factor de riesgo. Los siguientes resultados provienen del análisis de las 21 familias reclutadas para el proyecto originario más otra familia, reclutada con idénticos criterios de tamaño (38 individuos) y diagnosticada de trombofilia inexplicada, que resultó portadora de la mutación PT G20210A. Su adición en el análisis incrementó el poder estadístico de la muestra. La distribución de genotipos en la posición PT 20210 fue la siguiente: 388 individuos normales (G/G), 43 heterocigotos (G/A) y 4 homocigotos (A/A).

6.4.1. Ligamiento entre el polimorfismo PT G20210A y los niveles de protrombina

El análisis de ligamiento *(Almasy y Blangero 1998)* entre los niveles de protrombina y este marcador resultó en un LOD de 3.6 ($p = 0.000024$). Esto demuestra que en la región marcada por PT G20210A, efectivamente existe un QTL que influye en los niveles de protrombina.

Para descubrir la presencia de desequilibrio de ligamiento con un QTL cercano o bien excluirla (en cuyo caso la mutación PT G20210A se confirmaría como funcional *per se*) realizamos un análisis combinado de ligamiento y asociación:

1º Demostración de la asociación entre el genotipo PT20210 y los niveles de protrombina ($p = 0.0000001$). Es decir, en nuestra muestra, los niveles medios de protrombina son significativamente distintos según el genotipo

$$G/G = 123.6 \pm 1.8\ \%$$
$$G/A = 141.1 \pm 3.7\ \%$$
$$A/A = 167.7 \pm 8.4\ \%$$

2º Repetición del análisis de ligamiento, pero ahora incorporando (controlando) el genotipo PT 20210 de cada individuo como una covariable en el modelo *(Almasy y col 1999)*. En estas condiciones, el análisis de ligamiento explora si existen otros polimorfismos en la región influyendo a los niveles de protrombina. El LOD de este nuevo análisis fue 0.0. Es decir, al controlar el efecto que el genotipo ejerce sobre los niveles plasmáticos de protrombina, la señal genética de ligamiento anterior (LOD = 3.6) desaparece. Esto implica que la única fuente de variabilidad de los niveles de protrombina en esa región es el polimorfismo G/A en la posición 20210 y que no existen otras variantes genéticas influyendo en el fenotipo. En otras palabras, se descarta el desequilibrio de ligamiento como responsable de la asociación observada y se demuestra que la mutación PT G20210A es el único polimorfismo funcional en la región sobre los niveles plasmáticos de protrombina.

6.4.2 Estudio de ligamiento entre el polimorfismo PT G20210A y el riesgo de trombosis

Mediante un análisis de *ligamiento simple* (un marcador frente a un fenotipo) basado en los componentes de la variancia *(Almasy y Blangero 1998)* se obtuvo un LOD de 0.44 ($p = 0.077$) tan sólo sugestivo de posible ligamiento.

Basados en la potencial correlación entre los niveles de protrombina y el riesgo de trombosis, se realizó un análisis de *ligamiento bivariante*, utilizando ambos fenotipos

simultáneamente bajo la hipótesis de que el QTL ejerce efectos pleiotrópicos sobre los 2 fenotipos *(Williams y col 1999 b).*

Esto aumentó la señal de ligamiento con los niveles de protrombina, que previamente había sido de LOD = 3.6 (apartado 6.4.1):

LOD = 4.7 (p = 0.0000015)

También aumentó la señal respecto al riesgo de trombosis:

LOD = 2.43 (p = 0.0004), en comparación con el resultado previo (LOD = 0.44). Es decir, se observa un ligamiento claro entre el polimorfismo y el riesgo de trombosis.

En resumen, los datos aportados por el proyecto GAIT representan la primera evidencia genética directa de que el polimorfismo PT G20210A es un polimorfismo funcional con efectos sobre los niveles de protrombina y el riesgo de trombosis.

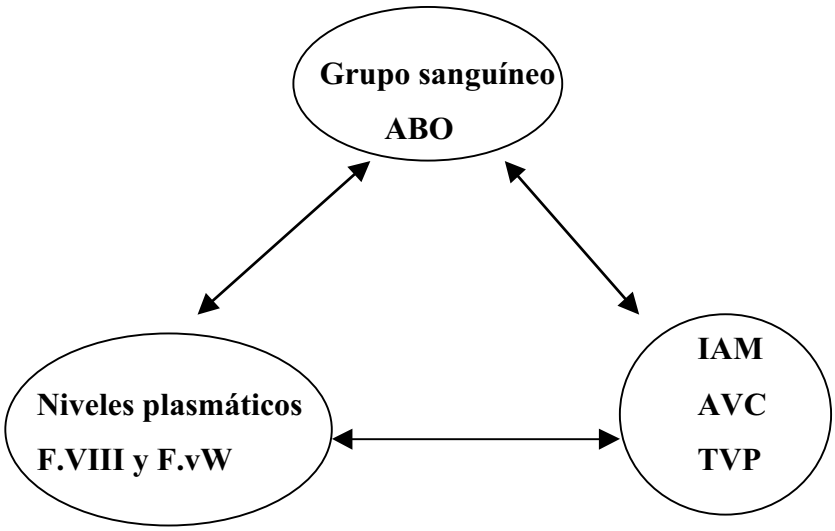
6.5 EL GRUPO SANGUÍNEO ABO Y SU RELACIÓN CON LOS FACTORES VIII Y VON WILLEBRAND

A principios de la década de los años 60 se describió la asociación entre el grupo sanguíneo ABO y la enfermedad arterial coronaria *(Medalie y col 1971)*. Desde entonces una gran cantidad de estudios, todos ellos caso/control han reportado la asociación de este polimorfismo genético con la enfermedad vascular tanto arterial *(Meade y col 1994)* como venosa *(O'Donnell y col 1997)*.

Por otro lado, el grupo sanguíneo ABO se asocia con los niveles en plasma de factor VIII y de factor von Willebrand *(Orstavik y col 1989)*.

Finalmente, los niveles en plasma de factor VIII y de factor von Willebrand se asocian con enfermedad cerebrovascular *(Mettinger 1982)*, trombosis venosa *(O'Donnell y col 1997, Koster y col 1995)* y enfermedad coronaria *(Meade y col 1994, Folsom y col 1997)*.

Existe pues, un triángulo de asociaciones, entre este marcador genético, estos fenotipos de la Hemostasia y las enfermedades vasculares:



De forma destacable, se observa una gran unanimidad en los resultados de todos los estudios publicados sobre este tópico: los individuos pertenecientes al grupo O presentan menor prevalencia de enfermedad vascular y también, niveles inferiores de factor VIII y factor von Willebrand. A su vez, los niveles aumentados de factor VIII y factor von Willebrand se asocian con un riesgo mayor de enfermedad.

Sin embargo, dadas las limitaciones metodólogicas de los estudios caso/control, a partir de los anteriores resultados y pese a su coherencia, es imposible:

- Establecer una relación causal inequívoca entre el grupo ABO, los niveles plasmáticos de ambos factores y el desarrollo de enfermedad vascular.

- Distinguir entre 2 posibilidades causantes de las asociaciones observadas. O bien el *locus* ABO (situado en el cromosoma 9) tiene un efecto funcional sobre los niveles plasmáticos. O por el contrario, lo que ocurre es que alguno de sus alelos se halla en desequilibrio de ligamiento con un alelo funcional situado en un *locus* cercano, distinto, pero ligado al ABO. Conviene precisar que el *locus* ABO contiene un solo gen, con variantes que codifican distintos enzimas glicosiladores. Los fenotipos eritrocitarios O, A, B y AB aparecen como resultado de las distintas cadenas de polisacáridos en las glicoproteínas de la membrana del hematíe *(Yamamoto y col 1990)*. Precisamente, el factor von Willebrand también es una glicoproteína y entre los oligosacáridos que contiene se encuentran algunos similares a los antígenos del grupo ABO *(Matsui y col 1992)*.

El diseño y los datos del estudio GAIT han permitido, por primera vez, aclarar la relación epidemiológica entre el marcador genético, las proteínas y la enfermedad. Esto es posible mediante análisis de ligamiento, usando el genotipo ABO, en lugar del fenotipo habitualmente utilizado en los estudios anteriores. El genotipo es más informativo porque permite distinguir entre 6 tipos de individuos (OO, OA, OB, AA, BB, AB) mientras que el fenotipo solo distingue 4 tipos (O, A, B, AB)

Se ha seguido una estrategia de análisis similar a la expuesta en el caso de la mutación PT G20210A (ver apartado 6.4.1):

El análisis de ligamiento *(Almasy y Blangero 1998)* entre el *locus* ABO y los niveles de factor von Willebrand resultó en un LOD de 2.19 ($p = 0.00075$).

El análisis de ligamiento entre el *locus* ABO y los niveles de factor VIII mostró una señal sugestiva con un LOD de 0.35 ($p = 0.10$).

Los niveles de factor VIII y de factor von Willebrand presentan una correlación genética significativa e intensa ($\rho_G = 0.703$, datos no publicados). Esto significa que tienen en común uno o varios genes reguladores. Como muy probablemente uno de ellos sea el gen ABO, se realizó un análisis de ligamiento bivariante, bajo la hipótesis de pleiotropía de este *locus* sobre ambos fenotipos *(Almasy y col 1997)*. Se comprobó una correlación intensa entre los efectos específicos de este *locus* sobre los 2 fenotipos ($p = 0.0005$). Por lo tanto, se puede muy razonablemente afirmar que el propio *locus* ABO o bien un *locus* ligado a él actúa pleiotrópicamente sobre los niveles de los factores VIII y von Willebrand.

A continuación se practicó un análisis de asociación que demostró niveles diferentes de proteína en función del genotipo ABO ($p = 0.0000001$ para von Willebrand; $p = 0.000008$ para Factor VIII). Es decir, también desde otra óptica distinta se confirma que el genotipo ABO es una variable que influye en los fenotipos.

Finalmente se practicó el análisis combinado de ligamiento / asociación. Se repitieron los análisis de ligamiento pero controlando el genotipo ABO como covariable en el modelo *(Almasy y col 1999)*. Si al controlar esta variable, se elimina toda evidencia de ligamiento (LOD = 0), entonces se puede concluir que el polimorfismo ABO es la única variable funcional en esa región causante del ligamiento:

	LOD ligamiento simple	LOD ligamiento/asociación
Factor von Willebrand	2.19	0.00
Factor VIII	0.35	0.00

En conclusión, el polimorfismo ABO es un determinante genético de los niveles de factor VIII y factor von Willebrand. Puesto que nuestros resultados previos también han demostrado la existencia de correlación genética entre estos factores y el riesgo de trombosis (Tabla 8), es altamente probable que el grupo sanguíneo ABO sea un determinante genético de la enfermedad tromboembólica.

6.6 IMPLICACIONES

Los resultados obtenidos después del análisis de los fenotipos en el proyecto GAIT tienen un alto valor teórico y práctico. Se ha demostrado una gran influencia de los genes sobre la variabilidad de todos los componentes de la Hemostasia y sobre el riesgo de sufrir la enfermedad tromboembólica. De hecho, la base genética de los individuos parece ser más determinante que las diferentes circunstancias ambientales para explicar las causas de la trombosis. La identificación final de los genes responsables y de sus variantes con efectos patológicos supondrá un enorme avance en el diagnóstico del riesgo individual. Hacia este objetivo se dirigen ahora los pasos del proyecto. Al reconocer las variantes genéticas realmente causantes de la patología será posible establecer perfiles según las distintas combinaciones de genotipos. Actualmente, la tecnología diagnóstica ya permite la realización rápida de centenares de marcadores genéticos distintos en un mismo individuo, con cantidades muy pequeñas de ADN (*microchips*). El problema, sin embargo, está en el contenido de estos análisis. Es muy importante seleccionar los polimorfismos con influencia patológica, y sólo ellos, porque de lo contrario se puede generar una confusión enorme. Baste recordar que, a día de hoy, tan solo se han identificado 3 polimorfismos relacionados claramente con la trombosis (factor V Leiden, mutación G20210A en el gen de la protrombina y grupo sanguíneo ABO). Sin duda, hay muchos otros, pero aún no se conocen.

La Medicina Molecular se encamina hacia los diagnósticos individualizados y también hacia los tratamientos específicos para cada enfermedad en cada individuo. Es aquí donde se situa la importancia del perfil genético en la batería de polimorfismos implicados en la trombosis. Este perfil determinará, en cada sujeto, las mejores estrategias profilácticas y también la elección de tratamientos más selectivos, dado que la respuesta a los fármacos también está regulada genéticamente.

De momento, no obstante, los resultados presentados en esta Tesis tienen unas implicaciones más modestas, aunque sin duda también importantes.

En el terreno teórico suponen un mejor conocimiento de la fisiología de la Hemostasia y de la patogenia de la enfermedad tromboembólica. Desde el punto de vista metodológico también se pueden extraer conclusiones muy interesantes. Se ha confirmado

que el diseño y los métodos estadísticos para el estudio de enfermedades o fenotipos complejos, aplicados en otras patologías como obesidad, alcoholismo, esquizofrenia o diabetes también son muy eficaces en el caso de la trombosis.

En el terreno práctico, algunos de los resultados pueden tener aplicación inmediata, fundamentalmente en el campo diagnóstico. A partir de ahora, ya tiene sentido incorporar otras determinaciones plasmáticas y genéticas al actual estudio biológico de trombosis en los pacientes con trombofilia. Los fenotipos que están correlacionados con el riesgo de trombosis tienen indudable valor clínico. Alguno de ellos, como la RPCa es habitual en la mayoría de laboratorios especializados. Otros, como la homocisteína o el factor VIII, se realizan de forma esporádica. Nuestros datos permiten sugerir las siguientes recomendaciones en la investigación de la trombofilia:

- Por supuesto, mantener la determinación de factores clásicos como la antitrombina, proteína C, proteína S y fibrinógeno. A pesar de que no parecen tener gran trascendencia en la trombosis común en la población general, pueden ser responsables de trombosis en familias seleccionadas, portadoras de mutaciones en los respectivos genes estructurales.
- Mantener el estudio de anticoagulante lúpico y anticuerpos antifosfolípido, como factores adquiridos de riesgo de trombosis.
- Determinar la RPCa además de estudiar su principal (pero no única) causa genética, el factor V Leiden.
- Determinar la mutación 20210 A en el gen de la protrombina y los niveles plasmáticos de protrombina.
- Determinar el grupo sanguíneo ABO, mediante genotipo o al menos mediante fenotipo eritrocitario convencional.
- Determinar niveles de factor VIII, factor von Willebrand y homocisteína.
- Valorar la introducción de otros factores de la Coagulación (VII, IX, XI y XII) y del t-PA. También el folato sérico merece ser considerado.
- En el futuro, muy probablemente el tiempo de protrombina mediado por trombomodulina puede ser de utilidad. Actualmente, esta técnica se halla en fase de estandarización y presentación de resultados. Todavía no existe método comercial.

7. CONCLUSIONES FINALES

1. La variabilidad de los fenotipos plasmáticos de la Hemostasia está determinada por los genes. En la mayoría de fenotipos el componente genético es muy intenso (heredabilidad > 30%). En muchos el componente genético es el principal, superior al componente ambiental (heredabilidad > 50%).

2. Algunos fenotipos exhiben un componente ambiental domiciliario, de menor intensidad (<20%), y que puede interpretarse, parcialmente, como efecto de la dieta.

3. El riesgo de enfermedad tromboembólica, en la población general española, está determinado genéticamente. Su heredabilidad es muy alta: 61 ± 16%. Esto significa que las diferencias en el riesgo de trombosis que presentan los individuos se explican en un 61% por las diferencias genéticas entre los sujetos y que un 39% se debe a sus diferentes condiciones ambientales.

4. Los genes involucrados en el riesgo de enfermedad tromboembólica venosa pueden ser en su mayoría los mismos que determinan la enfermedad tromboembólica arterial.

5. Varios de los fenotipos de la Hemostasia están correlacionados genéticamente con el riesgo de enfermedad tromboembólica. En otras palabras, los mismos genes que regulan su variabilidad, regulan el riesgo de trombosis. Se trata de RPCa, Factor VII, Factor VIII, Factor IX, Factor XI, factor XII, t-PA, factor von Willebrand, homocisteína, folato sérico y tiempo de protrombina mediado por trombomodulina.

6. Los genes que regulan la variabilidad de los fenotipos dependientes de la vitamina K (factor II, factor VII, factor IX, factor X, proteína C y proteína S) constituyen muy probablemente un grupo de pleiotropía.

7. La mutación en el gen de la protrombina (factor II) G20210A es funcional en relación con los niveles plasmáticos de protrombina y con el riesgo de trombosis.

8. El polimorfismo genético que determina el grupo sanguíneo ABO es funcional respecto a los niveles plasmáticos de factor VIII y de factor von Willebrand. A través de esta relación, es muy probable que el grupo ABO sea uno de los determinantes genéticos del riesgo de trombosis.

9. Los resultados anteriores (en especial los relacionados con los puntos 1, 3, 4 y 5) justifican plenamente la realización de proyectos más ambiciosos y costosos como el Análisis Global del Genoma en busca de los genes implicados.

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