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

Juan Carlos Souto, Laura Almasy, Eduardo Muñiz-Diaz, José Manuel Soria, Montserrat Borrell, Laia Bayén, José Mateo, Pedro Madoz, William Stone, John Blangero, Jordi Fontcuberta

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.5,15 In addition, >15 reports have established an association between the ABO blood group and plasma levels of factor VIII6–9 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,3,14–16 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) Project21 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 \( \geq 1 \) 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
linked to the structural loci on human chromosome 12\(^2\) and the X chromosome,\(^3\) 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.\(^4\) For example, factor VIII coagulant activity (factor VIII:C) has 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.\(^4\) 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 2000g 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 quantitated 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 group procedures.

DNA was extracted from EDTA blood by use of a salting-out method\(^5\) or a commercial kit (Wizard, Promega Corp.). ABO genotyping was performed by polymerase chain reaction (PCR) as described\(^6\) with use of the following primers: for exon VI, mo-46 5′-CGGATTTCACTCGCCACTGTGCCTGCCTC-3′ and mo-57 5′-CGGATCTCGTGCGTCCTGCA-3′; and for exon VII, mo-101 5′-CGGGATCCCGTGCGTCCTGCA-3′ and mo-71 5′-GGCCCTAGGCCTGATGTACTC-3′.

PCR was performed in a total volume of 10 µL under the following conditions: initial denaturation at 94°C for 2 minutes, followed by 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 µL 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 HpaII and KpnI (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.\(^7\) 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 (i.e., 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.\(^8\)

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.\(^9\) 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

<table>
<thead>
<tr>
<th>ABO Phenotype</th>
<th>Gross Genotype</th>
<th>Specific Genotype</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group O (N=139)</td>
<td>O/O</td>
<td>O/O</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>O/O</td>
<td>O/O</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>O/O</td>
<td>O/O</td>
<td>0</td>
</tr>
<tr>
<td>Group A (N=211)</td>
<td>A/O</td>
<td>A/O</td>
<td>151</td>
</tr>
<tr>
<td></td>
<td>A/O</td>
<td>A/O</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A/O</td>
<td>A/O</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>AA</td>
<td>33</td>
</tr>
<tr>
<td>Group B (N=31)</td>
<td>B/O</td>
<td>B/O</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>B/O</td>
<td>B/O</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>BB</td>
<td>BB</td>
<td>0</td>
</tr>
<tr>
<td>Group AB (N=10)</td>
<td>A/B</td>
<td>A/B</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>A/B</td>
<td>A/B</td>
<td>0</td>
</tr>
</tbody>
</table>

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. 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.

Results

Linkage analyses were performed with the use of ABO genotypes (Table 1), which distinguish the O1 and O2 alleles and the A2 and A2 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 A2 and O2 alleles, we grouped alleles A1 and A2 into A and O1 and O2 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 genotypes 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 × 10⁻⁷; factor VIII:C, P = 8.2 × 10⁻⁶; 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.

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. 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. used molecular methods to precisely delineate the ABO genotype in relation to vWF levels. The ability to distinguish the A1 and A2 alleles and the O1 and O2 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, including molecules similar to blood group A, B, and H (O) antigens. 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. 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. Additionally, studies of candidate genes involved in hemostasis have identified functional polymorphisms that influence the quantitative phenotypes and risk of thrombosis. A recent association study has yielded further evidence that the vWF structural locus influences vWF levels. Interestingly, recent work on a mouse model has revealed a modifier locus involving lineage-specific expression of a glycosyltransferase that also influences vWF levels. 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.

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References


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