ABO Blood Groups and Genetic Risk Factors for Thrombosis in Croatian Population

**Aim** To assess the association between ABO blood group genotypes and genetic risk factors for thrombosis (FV Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations) in the Croatian population and to determine whether genetic predisposition to thrombotic risk is higher in non-OO blood group genotypes than in OO blood group genotypes.

**Methods** The study included 154 patients with thrombosis and 200 asymptomatic blood donors as a control group. Genotyping to 5 common alleles of ABO blood groups was performed by polymerase chain reaction with sequence specific primers (PCR-SSP). FV Leiden was determined by PCR-SSP, while prothrombin and methylenetetrahydrofolate reductase were determined by PCR and restriction fragment length polymorphism (PCR-RFLP).

**Results** There was an association between non-OO blood group genotypes and the risk of thrombosis (odds ratio [OR] 2.08, 95% confidence interval [CI], 1.32-3.27). The strongest association with thrombotic risk was recorded for A1B/A2B blood group genotypes (OR, 2.73; 95% CI, 1.10-6.74), followed by BB/O1B/O2B (OR, 2.29; 95% CI, 1.25-4.21) and O1A/O2A1 (OR, 1.95; 95% CI, 1.15-3.31). FV Leiden increased the risk of thrombosis 31-fold in the group of OO carriers and fourfold in the group of non-OO carriers. There was no significant difference in the risk of thrombosis between OO and non-OO blood groups associated with prothrombin mutation. Non-OO carriers positive for methylenetetrahydrofolate reductase had a 5.7 times greater risk of thrombosis than that recorded in OO carriers negative for methylenetetrahydrofolate reductase.

**Conclusion** Study results confirmed the association of non-OO blood group genotypes with an increased risk of thrombosis in Croatia.
ABO gene is located on chromosome 9 and its inheritance is explained by the Mendel and Bernstein three-allele theory. Methods of serologic typing allow the determination of 6 main phenotypes: A, B, A2, A2B, AB, and O. Methods of molecular biology allow the differentiation of 5 common alleles: O, O, A, A, and B; and 15 genotypes. ABO system is very well characterized by more than 165 alleles. Population studies have documented different frequencies of particular ABO genotypes worldwide (1). Also, there have been reports on a lower or higher association between ABO blood groups and cardiovascular, gastrointestinal, and infectious diseases (2).

The association of ABO blood groups and diseases resulting in coagulation impairment and venous thrombus formation was first described by Jick et al (3). Most studies performed to date have generally agreed that non-OO blood group carriers have a higher risk of thrombosis than OO blood group carriers (4-7). Wu et al (8) believe that the introduction of ABO typing in the evaluation of thrombophilia patients should be considered in A/A/A/B and BB genotypes with elevated von Willebrand factor (vWF) antigen levels in particular.

Bezemer and Rosendaal (9) investigated new predictive genetic variants for venous thrombosis and identified ABO blood groups, fibrinogen gene haplotypes, factor V, factor VIII, and factor XIIVal34Leu polymorphisms as the possible new predictive variants for the disease development.

Blood group O shows a tendency to bleeding and blood group A to clotting, due to the higher level of coagulation factors VIII and vWF in these individuals. The ABO blood groups also determine plasma levels of vWF, which is by 25-30% lower in O blood group than in non-O blood group. This occurs due to the direct functional impact of ABO locus; however, the exact mechanism has not yet been elucidated. Theoretically, ABO blood group can modify the rate of vWF synthesis or its secretion within endothelial cells, or act upon vWF catabolism, ie, proteolysis and/or plasma clearance (10).

Venous thrombosis and disorders associated with coronary artery disease are vascular diseases in which both inherited and acquired factors are involved. It is of utmost importance to identify the risk factors implicated in the disease development and the candidates eligible for anticoagulant therapy to prevent recurrent thrombotic episodes. The most common inherited prothrombotic risk factors with significant impact on the development of thrombosis are mutations of the genes encoding proteins involved in the coagulation cascade, such as FV Leiden mutation and G20210A mutation of the prothrombin gene (11). In addition to coagulation factors, mutation of the methylene-tetrahydrofolate reductase gene, known to influence the development of atherosclerosis and vascular diseases, has also been extensively investigated (12). It should be noted that the thrombogenic impact of homozygosity for methylene-tetrahydrofolate reductase C677T mutation is only pronounced in case of its association with mild or moderate hyperhomocysteinemia and the genetic mutations mentioned above (13,14).

The aim of this study was to define ABO genotypes in the population of Croatian blood donors and to assess the impact of ABO blood group genotypes and FV Leiden, prothrombin G20210A, and methylene-tetrahydrofolate reductase C677T mutations on thrombosis development in the Croatian population. In addition, we investigated whether predisposition to thrombosis was higher in non-OO genotype carriers than in OO genotype carriers. Genotyping at 5 common alleles (O, O, A, A, and B) was used for ABO blood group determination because it provides more information on homozygous and heterozygous carriers of A, B, and O alleles than serotyping. The risk of thrombosis caused by a combination of multiple genetic factors (interaction of 2 risk factors) was also assessed.

PARTICIPANTS AND METHODS

Participants

A total of 154 out-patients treated at Coagulation Department, Croatian Institute of Transfusion Medicine, Zagreb, Croatia, from 2005 until 2007 were included in the study. They were referred to our institution for an evaluation of hemostatic parameters with disease follow-up. There were 100 female and 54 male patients with median age of 46 years (18-86 years); 74 and 80 patients were aged ≤45 and >45, respectively. According to the diagnosis made on the initial thrombotic episode by ultrasonography, venography/phlebography, coagulation tests, and history data, patients were divided into 3 subgroups: venous thromboembolism (VTE) (n=60, 36 female/24 male, median age 46 years, range 18-86); chronic venous diseases (CVD) (n=69, 48 female/21 male, median age 50 years, range 19-81); and arterial thrombosis (n=25, 16 female/9 male, median age 39 years, range 20-64). The VTE subgroup included patients with deep vein thrombosis and pulmonary embolism. The CVD subgroup included patients with thrombophlebitis and varicosity. The arterial thrombosis...
subgroup included patients with thrombosis of the intra-arterial vascular system such as stroke, myocardial infarction, and atrial fibrillation, and those with coagulation and circulation disorders.

Control group comprised 200 asymptomatic and healthy blood donors (87 female/113 male) without history of thrombosis and free from vascular diseases during the 2005-2006 period. Median age of control participants was 40 years. All participants in the control group were from the same geographic region; there were 119 and 81 participants aged ≤45 and >45, respectively.

Data on age, sex, and genetic factors for thrombosis in the patient and control group are summarized in Table 1. Whereas patient group showed a female predominance (100 female vs 54 male), in control group the sex ratio was more balanced (87 female vs 113 male). The groups with VTE, CVD, and arterial thrombosis did not differ significantly with respect to age (median test; overall median, 46.0, χ²=3.375, P=0.185; Kruskal Wallis analysis of variance, H=2.862, P=0.239). However, patient groups were significantly older than the control group (median test; overall median, 43.5, χ²=10.328, P=0.016; Kruskal Wallis analysis of variance, H=16.068, P=0.001), particularly VTE and CVD groups that were significantly older than control group (median test, VTE vs control, P=0.005, CVD vs control P=0.016).

In control group, the distribution of ABO blood group phenotypes was as follows: group O – 86 (43%); group A – 72 (36%); group B – 32 (16%); and group AB – 10 (5%) participants. The procedures used in the study were in accordance with ethical standards of the responsible committee on human experimentation (institutional or regional) or with the 2008 revision of the Helsinki Declaration. An informed consent was obtained from all study participants prior to entering the study.

**ABO genotyping by polymerase chain reaction with sequence specific primers**

Genomic DNA isolation from EDTA blood samples was performed by the commercial spin column procedure QiaAmp DNA Mini kit (Qiagen, Hilden, Germany). ABO genotypes were determined in 8 parallel PCR-SSP reactions to amplify exons 6 and 7 of the ABO blood group gene, according to Gassner (15), with some modifications in primer dilution and PCR conditions. For each PCR assay, a master mix (20.0 μL) was prepared consisting of 2.0 μL of 10×PCR buffer +1.5 mM MgCl₂ and 0.8 μL of 2 nM dinucleotide triphosphate mix (0.25 μL each). The concentration of specific primers was 1 μM, concentration of internal control (human growth hormone) 0.1 μM, bidistilled H₂O, 0.1 μL, Taq polymerase (AmpliTaq DNA polymerase, Applied Biosystems, Branchburg, NJ, USA) 5 U/μL, and 1.5-2.0 μL of a solution of 10 μg/mL DNA.

The PCR protocol was carried out under the following conditions: denaturation of DNA at 96°C for 2 minutes, 5 cycles – at 96°C for 25 seconds, 70°C for 50 seconds, and 72°C for 45 seconds, 25 cycles – at 96°C for 25 seconds, 65°C for 50 seconds, and 72°C for 45 seconds, and additional 10 cycles – at 96°C for 25 seconds, 61°C for 50 seconds, and 72°C for 45 seconds.

PCR products (20 μL) were run on a standard 2% agarose gel. The presence of gene fragment of the human growth hormone (internal control) in each SSP reaction showed that amplification was successful. The ABO alleles and gen-

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. (%) of patients with</th>
<th>No. (%) of patients with</th>
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<tbody>
<tr>
<td></td>
<td>women</td>
<td>men</td>
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<tr>
<td>Venous thromboembolism (n=60)</td>
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<td>24 (40)</td>
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<td>48 (69.6)</td>
<td>21 (30.4)</td>
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<tr>
<td>Arterial disease (n=25)</td>
<td>16 (64)</td>
<td>9 (36)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (64.9)</td>
<td>54 (34.1)</td>
</tr>
<tr>
<td>Control group (n=200)</td>
<td>87 (43.5)</td>
<td>113 (56.5)</td>
</tr>
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</table>

*Abbreviations: FVL – factor V Leiden; FII – prothrombin; MTHFR – methylenetetrahydrofolate reductase.
otypes were named according to the nomenclature used by Yamamoto (1).

**Analysis of FV Leiden mutation by PCR-SSP method**

FV Leiden genotyping was performed by PCR-SSP amplification of extracted DNA in two separate reactions, one containing primers for the wild type allele and the other containing primers for the mutant allele (16). PCR products (20 μL) were run on a standard 1.5% agarose gels. PCR product for wild type is 149 bp and for mutant allele 174 bp. Internal control for co-amplification in each SSP reaction was β-actin gene fragment of 310 bp.

**Analysis of prothrombin G20210A mutation by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP)**

The prothrombin G20210A mutation was identified by PCR amplification, followed by RFLP digestion (17). PCR product of mutated site was digested with Hind III in the 10X buffer (Invitrogen, Carlsbad, CA, USA). The digestion mix (20 μL) containing 2 U of Hind III and 10 μL of PCR product was incubated for 3-4 h at 37°C. Restriction products were separated on 3% agarose gel. Participants with normal genotype had a wild type fragment of 345 bp, homozygous carriers of mutant allele were identified by the presence of a 322 bp fragment, and heterozygous carriers had both alleles, yielding both fragments on agarose gel (17).

**Analysis of methylenetetrahydrofolate reductase C677T mutation by PCR-RFLP method**

The methylenetetrahydrofolate reductase C677T mutation was amplified using primers described by Frosst et al (18), where the PCR fragment was digested by Hinf I. The PCR product of mutated site was digested with Hinf I in 10X buffer (Invitrogen, Carlsbad, CA, USA). The digestion mix (20 μL) containing 2 U of Hinf I and 10 μL of PCR product was incubated for 3-4 h at 37°C. Restriction products were separated on 3% agarose gel. Normal genotype was identified by the presence of a wild type fragment of 198 bp, whereas mutant homozygous carriers were identified by the presence of a 175-bp fragment and heterozygous carriers by the presence of both fragments (18).

**Statistical analysis**

Results were obtained by calculation of odds ratio (OR) of the data from contingency tables (incidences), with 95% confidence interval (95% CI) calculation based on large sample approximation to the sampling distribution of the log odds ratio. OR calculation was used to assess the impact of combinations of ABO blood group genotypes and genetic risk factors on thrombosis development. Fisher exact test was used in the analysis of contingency tables with small sample sizes. The level of statistical significance was set at 0.05 in all analyses (MedCalc software version 4.10 for Windows 95, MedCalc Software; Mariakerke, Belgium).

**RESULTS**

**Prevalence of genetic factors for thrombosis**

Thirty-two heterozygous and 2 homozygous carriers of FV Leiden were found in the patient group as opposed to only 7 heterozygous carriers in the control group. Also, 8 and 7 heterozygous carriers of prothrombin G20210A mutation, and 26 and 21 homozygous methylenetetrahydrofolate reductase carriers were recorded in the patient and control group, respectively.

**Prevalence of ABO genotypes**

Three homozygous ABO genotypes, ie, O\textsuperscript{2}O\textsuperscript{2}, A\textsuperscript{2}A\textsuperscript{2}, and BB, were not found in the patient and control group, whereas other 3 genotypes, O\textsuperscript{1}A\textsuperscript{2}, O\textsuperscript{2}B, and A\textsuperscript{2}B, were very rare and were only present in the patient group (Table 2).

**OO and non-OO genotype carriers and risk of thrombosis**

Non-OO blood group carriers from both patient and control group had higher probability of developing

<table>
<thead>
<tr>
<th>Blood group</th>
<th>Genotype</th>
<th>No. (%) of patients</th>
<th>No. (%) of controls</th>
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<tr>
<td>O</td>
<td>O\textsuperscript{0}O\textsuperscript{0}</td>
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<td></td>
<td>O\textsuperscript{1}A\textsuperscript{1}</td>
<td>3 (1.9)</td>
<td>3 (1.5)</td>
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<tr>
<td></td>
<td>O\textsuperscript{1}A\textsuperscript{2}</td>
<td>1 (0.6)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td></td>
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<td>2 (1.0)</td>
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<td></td>
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<td>1 (0.6)</td>
<td>1 (0.5)</td>
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<tr>
<td>B</td>
<td>O\textsuperscript{1}B</td>
<td>34 (22.2)</td>
<td>32 (16)</td>
</tr>
<tr>
<td></td>
<td>O\textsuperscript{0}B</td>
<td>1 (0.6)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>A\textsuperscript{1}B</td>
<td>1 (0.6)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>A\textsuperscript{0}B</td>
<td>1 (0.6)</td>
<td>–</td>
</tr>
<tr>
<td>AB</td>
<td>A\textsuperscript{1}B</td>
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<td>10 (5)</td>
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<td></td>
<td>A\textsuperscript{0}B</td>
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</tr>
<tr>
<td>n</td>
<td>154</td>
<td>200</td>
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thrombosis than OO-carriers (OR, 2.08; 95% CI, 1.32-3.27, P=0.001).

Genotype comparison between non-OO and OO carriers revealed genotypes with A and B alleles to pose a slightly elevated thrombotic risk. OR was 1.95 (95% CI, 1.15-3.31) for O'A/O'A' genotypes; 2.29 (95% CI, 1.25-4.21) for BB/O'B/ O'B genotypes; and was highest for A'B/A'B genotypes (OR, 0.06-4.84), and O'A'/O'A'/A'A' genotypes (OR, 1.68; 95% CI, 0.43-6.58) were not found to be significant risk factors for thrombosis (Table 3).

Combined effect of OO and non-OO blood group carriers and genetic factors for thrombosis

Table 4 shows the comparison of OO and non-OO blood group carriers of FV Leiden, FII prothrombin (G20210A), and methylenetetrahydrofolate reductase (677TT) as genetic factors for thrombosis. In the group of OO carriers, the presence of FV Leiden mutation increased the risk of thrombosis (OR, 31.2; 95% CI, 3.9-251.7). Comparison of OO carriers without FV Leiden mutation and non-OO carriers with FV Leiden mutation showed the risk of thrombosis to be tenfold in the latter (OR, 9.9; 95% CI, 3.7-26.9). Only FV Leiden heterozygous carriers were included in the calculations because there were no homozygous carriers in control group to be compared with 2 homozygous carriers in patient group. In contrast to FV Leiden mutation, the presence of prothrombin gene G20210A mutation had no significant impact on the development of thrombosis in either OO or non-OO carriers. Testing the thrombogenic effect of homozygosity for methylenetetrahydrofolate reductase C677T mutation indicated the non-OO carriers with methylenetetrahydrofolate reductase C677T mutation to be at a twofold risk of thrombosis compared with non-OO carriers without methylenetetrahydrofolate reductase C677T mutation (OR, 1.90; 95% CI, 1.20-3.24). Although a few tests resulted with poor statistical power, the aver-
The results of the present study indicate the non-OO blood group carriers to be at a significant, twofold risk of thrombosis compared with OO blood group carriers. Genotype analysis of non-OO carriers showed the highest risk of thrombosis in carriers of AB and A^2B genotypes, followed by BB/O^1B/O^2B genotypes and O^1A/O^2A genotypes. Accordingly, the significant risk of thrombosis was higher in the carriers of B alleles than in those carrying A alleles. Based on the study results, A^2 allele did not appear to be a thrombotic risk factor, as demonstrated in A^1A^2 (OR 0.52) and O^1A/O^2A/O^3A (OR 1.68) genotypes. Yet, A^2 allele was present in A^B genotype, which is considered as a thrombotic risk factor due to the presence of B allele. Unfortunately, the number of patients and controls with some rare genotypes was too small to draw definite conclusions on these associations. The Netherlands’ large LETS study performed by Morelli et al (19) showed the non-OO genotype to be a significant risk factor for venous thrombosis, with OR 1.8 (95% CI, 1.4-2.4), which is comparable to our results (OR, 2.08). They concluded that all non-OO genotypes except A^2 homozygotes or O^1A^2 combinations were associated with an increased risk of thrombosis, which is consistent with our results, with the exception of A^1A^2 genotype. Similarly, a meta-analysis of studies on the association between vascular disease and ABO showed pooled OR for venous thromboembolism to be 1.79 (8). Studies on arterial thrombotic diseases reported similar but less pronounced association with non-OO blood groups as studies on venous thrombosis or even failed to confirm it. In a study on the association of ABO system and acute myocardial infarction in Switzerland, Nydegger et al (7) found B allele to be an independent risk factor for myocardial infarction (OR, 2.7). In addition, homozygous carriers of BB blood group showed the highest level of vWF, which is known to increase the risk of myocardial infarction. B antigen additionally contributes to the increased platelet aggregation. Wu et al (8) defined pooled OR of 1.25 for myocardial infarction and 1.14 for cerebral ischemia of arterial origin. In a study including Iranian patients with coronary artery disease, Amirzadegan et al (4) found no association with particular ABO blood groups.

Our results showed the presence of FV Leiden mutation to increase the risk of thrombosis by as many as 31 times in OO genotypes and about 4 times in non-OO genotypes. Too high OR (31.2) in OO genotypes is a consequence of having only one person with FV Leiden mutation in the control group and a wide confidence interval is a consequence of the small sample size. If two persons had had FV Leiden mutation, the OR would have been cut in half. This shows the limitation of statistical analysis.
used on a small number of study participants. In non-OO carriers positive for FV Leiden mutation, the risk of thrombosis was tenfold compared with that recorded in OO carriers negative for FV Leiden mutation. According to Morelli et al (19), OO carriers negative for FV Leiden in combination with OO FV Leiden positive carriers had an increased risk of thrombosis, with OR 4.6 (95% CI, 2.0-10.1), whereas a combination of OO FV Leiden negative carriers with non-OO FV Leiden positive carriers yielded OR 23.2 (95% CI, 9.1-59.3). Despite different OR values obtained in our study and those reported in the studies above, the conclusion is identical, ie, that this combination of ABO blood group genotype and FV Leiden carrier state is a risk factor for the development of thrombosis. The Procare-GEHT (20) group investigated non-OO blood groups as risk factors for the development of thrombosis in homozygous FV Leiden carriers (OR, 4.1, 95% CI, 1.9-8.9). The prevalence of non-OO blood groups in patients with venous thromboembolism and control participants was assessed in the Longitudinal Investigation of Thromboembolic Etiology study (21). The authors reported on OR, 1.64 (95% CI, 1.32-2.05) in patients with venous thromboembolism and non-OO blood groups. The presence of FV Leiden additionally increased OR to 6.77. Interestingly, the Afro-Americans included in the study had a higher proportion of OO blood group and greater prevalence of venous thromboembolism, suggesting that some risk factors other than blood group, eg, obesity, diabetes mellitus, and elevated factor VIII level, also contribute to thrombotic risk (21).

In their meta-analysis, Wu et al (8) found OR of 3.88 for non-OO and FV Leiden carriers, and none of the studies included in the analysis had demonstrated an increasing effect of prothrombin 20210A mutation on the thrombotic risk. In our study, 20210A prothrombin gene mutation was not found to pose a significant risk factor in combination with OO and non-OO blood groups either. The OO carriers of prothrombin mutation did have a 6.7-fold predisposition to thrombosis development, but it was not significant. The non-OO carriers showed an even lower, non-significant potential to develop thrombosis (OR, 0.83). Similar results reported by Ordonez-Gonzalez et al (22) also suggest that this prothrombin gene mutation does not increase the risk of thrombosis.

Very informative are the results of study of Minano et al (23), which has analyzed the effect of genotypes of ABO blood groups on venous or arterial thrombosis in a great number of carriers (n=981) of FVL or prothrombin G20210A polymorphism. They reported that non-OO blood group did not increase the risk of myocardial infarction in carriers of FVL or prothrombin G20210A. Alternatively, their results confirmed the previously documented risk of VTE among FVL carriers who had a non-OO blood group (OR, 1.76). This is the first large study suggesting that non-OO blood group increases the risk of VTE in carriers of prothrombin polymorphism.

Our study has some limitations, such as the relatively small number of patients and controls, due to the non-existence or low frequency of prothrombotic carriers among participants. Additionally, the patient group is heterogenous with respect to presence of arterial thrombosis. However, taking into account only the subgroup of patients with venous thrombosis, the conclusions on association of non-OO blood genotypes and prothrombotic polymorphisms is valid.

In this study, we also investigated the effect of methylenetetrahydrofolate reductase C677T mutation in homozygous carriers, since heterozygous carrier state is not associated with an increased risk of thrombosis. When OO/methylenetetrahydrofolate reductase negative carriers were compared with OO/methylenetetrahydrofolate reductase positive carriers, this combination of risk factors was not found to be significant.

Isolated methylenetetrahydrofolate reductase C677T mutation is not a risk factor for thrombosis unless being associated with some other genetic or acquired risk factor, as demonstrated in our study as well as in other related studies (9,14,20).

The main conclusion of this study is that carriers of ABO blood group non-OO genotype have a slightly elevated twofold predisposition to develop thrombosis compared with that found in OO genotypes. FV Leiden mutation carriers are at an additional thrombotic risk, higher in OO blood group carriers.

ABO blood group genotyping in blood donors could produce valuable results for use in epidemiologic and anthropologic studies in our population and which could serve as a basis for future research on the association between ABO system and various diseases.

In this study, the criteria for valid statistical analysis in terms of adequate and properly selected study population were met. Study results defined ABO system genotypes showing significant correlation with the development of venous
and arterial thromboembolism, thus providing a valuable tool for the prevention and target management of these pathologies. We believe that these results will greatly contribute to making proper decision whether ABO genotyping should be included in the mandatory risk testing in thromboembolism.

Acknowledgments

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References

