Background and Objectives. The aims of this study were to compare the lifetime probability of developing thrombosis in 722 relatives of 132 thrombophilic families of symptomatic probands with recognized thrombophilic defects and to determine the prevalence of the factor V Leiden (FVL) mutation and the 20210A allele of the prothrombin gene (PT20210A) in these families.

Design and Methods. The study included 722 members belonging to 132 unrelated families. The propositi were patients who had been referred to our Thrombosis Unit. The families were selected through a symptomatic proband. Once a patient with a deficiency or mutation was identified, family members were screened for the same defect.

Results. The prevalence of FVL and PT20210A in families with other thrombophilic defects was higher than expected. Compared with non-deficient individuals, the risk of venous thrombosis was increased in subjects with antithrombin (AT), protein S (PS) and protein C (PC) deficiencies, and in carriers of FVL and PT20210A mutations. The risk of thrombosis was significantly increased for individuals with combined genetic defects (PC-FVL, PS-FVL, PS-PT20210A and FVL-PT20210A). The ages at the time of 50% thrombosis-free survival were as follows: 34 years for AT deficiency, (19 years with FVL, 21 years with PT20210A), 62 years for PC deficiency (33 years with FVL, 44 years with PT20210A), 37 years for PS deficiency (24 years with FVL, 36 years with PT20210A), 50 years for the FVL mutation (52 years with PT20210A), and 65 years for the PT20210A mutation. As for clinical characteristics, no differences were observed except for the higher frequency of oral contraceptive-related thrombosis in women who were carriers of PT20210A or FVL.

Interpretation and Conclusions. Based on these results, screening for FVL and PT20210A mutation is recommended in patients with other thrombophilic defects. To the best of our knowledge, this is the first family study, including the PT20210A mutation, that compares genetic risk factors for thrombosis and the lifelong probability of developing thrombosis.

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Key words: thrombophilia, genetic risk factors, PT20210A mutation, factor V Leiden, family study.

Venous thrombosis is the most common cardiovascular disorder after ischemic heart disease and stroke. It is caused by both environmental and genetic factors. The high prevalence of thrombosis and its environmental influences (e.g. oral contraceptive use) suggest that multiple genes with varying effects are involved in determining susceptibility to this complex disease. There are a number of well-characterized genetic defects that lead to increased thrombotic risk. Of these, the factor V Leiden (FVL) mutation in the coagulation factor V gene, and the G20210A mutation in the prothrombin gene (PT20210A) are the most prevalent genetic risk factors that predispose individuals to a substantially increased risk of venous and arterial thromboses. Double-heterozygosity for these mutations is the most common combined condition associated with thrombophilia. Moreover, these mutations are often
associated with other genetic defects that predispose to thrombotic disease, such as protein C (PC) and protein S (PS) deficiencies. These combined defects confer a higher risk of thrombosis than either defect alone. Consequently, it has been postulated that more than one genetic risk factor may co-segregate to cause a cumulative or synergistic effect on thrombotic risk. 

To investigate such a complex disorder, two strategies have been used: clinical case-control studies and genetic epidemiologic studies. The latter strategy has considerable utility since the evaluation of large families is a most robust application. In fact, the role of all known prothrombotic risk factors in the clinical course of thrombophilia has been compared in a number of family studies and has been discussed in several reports. How- ever, only one such report considered FVL mutation and none considered the role of the PT20210A mutation. Because knowledge of any difference in the thrombotic risk might have implications for patient management strategies, and because the PT20210A mutation is the most prevalent genetic risk factor for thrombosis in the Spanish population, we performed a retrospective family study of 722 affected and unaffected members from 132 Spanish families with inherited deficiency of AT, PC, PS, or with FVL and PT20120 mutations.

Design and Methods
The study included 722 members belonging to 132 unrelated families. The propositi were patients who had been referred to our Thrombosis Unit. The families were selected through a symptomatic proband. Once a patient with a deficiency or mutation was identified, family members were screened for the same defect. In the case of PS deficient families in which both type I and type III deficiencies were present, the family was classified according to the predominant phenotype. Eleven families with AT deficiency (10 type I and 1 with type II deficiency with decreased affinity of antithrombin for heparin and loss of activity, pleitropic effect), 34 with PC deficiency, 40 with PS deficiency (13 type I and 27 type III), 32 with the FVL mutation and 15 with the PT20210A mutation were included in the analysis (Table 1). In 4 out of 13 type I PS deficient families, there were members with type III PS deficiency. In these four families, 48 individuals were screened (22 had type I, 6 had type III and the other 20 were normal). As for type III, 4 out of 27 families had members who exhibited the type I phenotype. In these four families, 48 individuals were screened (22 had type I, 6 had type III and the other 20 were normal). As for type III, 4 out of 27 families had members who exhibited the type I phenotype. In these four families, 30 individuals were analyzed (7 had type III, 4 type I and 19 did not have a protein deficiency).

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A medical history was taken with special emphasis on the localization of thrombosis and on the presence of acquired predisposing factors. The diagnosis of deep venous thrombosis of the lower limbs was objectively established by ultrasonography or ascending venography. Pulmonary embolism was diagnosed by ventilation-perfusion lung scan

Table 1. Prevalence of FVL and PT20210A mutations in families with other thrombophilic defects. Thrombosis and asymptomatic individuals classified by type of deficiency and thrombosis risk of each deficiency (expressed as adjusted odds ratio and 95% CI).

<table>
<thead>
<tr>
<th>Deficiencies</th>
<th>no. of families (%)</th>
<th>Thrombosis</th>
<th>odds ratio (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no. of individuals)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>No defect</td>
<td>8 (27.9%)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Antithrombin (n=11)</td>
<td>8 (72.7%)</td>
<td>2 (18.2%)</td>
<td>1</td>
</tr>
<tr>
<td>+FVL</td>
<td>1 (9.1%)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>+PT20210A</td>
<td>1 (9.1%)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Protein C alone (n=34)</td>
<td>22 (64.7%)</td>
<td>29</td>
<td>54</td>
</tr>
<tr>
<td>+FVL</td>
<td>5 (14.7%)</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>+PT20210A</td>
<td>7 (20.6%)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Protein S alone (n=40)</td>
<td>31 (77.5%)</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>+FVL</td>
<td>4 (10.0%)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>+PT20210A</td>
<td>5 (12.5%)</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>FV Leiden alone (n=32)</td>
<td>26 (81.2%)</td>
<td>32</td>
<td>51</td>
</tr>
<tr>
<td>+PT20210A</td>
<td>6 (18.8%)</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>PT20210A (n=15)</td>
<td>15</td>
<td>20</td>
<td>69</td>
</tr>
</tbody>
</table>

FVL: factor V Leiden mutation; PT20210A: 20210A mutation of the prothrombin gene. *Adjusted for age, sex and type of individual (propositus or relative).
nong or pulmonary angiography. Thrombosis at other unusual sites was diagnosed by computed tomography, magnetic resonance imaging, ophthalmoscopic examination or as a surgical finding depending on the type of thrombosis and on its localization. The patients were asked whether they had suffered from previous episodes of venous thromboembolism. If the answer was affirmative, the age at the time of the first episode, the localization of the thrombosis and the recognized predisposing factors were recorded. The episode was considered spontaneous in the absence of triggering risk factors. Information about thrombosis was obtained from some family members using a questionnaire similar to that used by Frezzato et al. As for non-objectively confirmed thrombotic events, we considered thrombosis as beyond doubt only when anticoagulant treatment had been given or signs of a post-phlebitic syndrome had been detected. This approach should have minimized the bias. Families with associated FVL and PT20210A were considered in the analyses as having FVL plus PT20210A.

Laboratory methods
Blood collection. Blood was collected 3 to 6 months after the most recent thrombotic event in the patients with thrombosis. Oral anticoagulants were withdrawn and the samples were taken after a washout period of at least 20 days. Blood samples were collected from the antecubital vein and immediately anticoagulated with 1/10 volume of 0.129-M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2,000 g for 20 min and was frozen and stored at -40°C until analysis.

Laboratory determinations. The following phenotypes were measured as previously described: functional antithrombin; functional protein C; functional, free and total protein S. To reduce measurement error all assays were performed in duplicate, and the average value was calculated for each person. Antigenic measurements of antithrombin and protein C were performed only if the functional assays were below the normal range. Intra-assay and inter-assay coefficients of variation were generally estimated between 2% and 6%.

Genetic analysis. Genomic DNA was isolated from peripheral blood leukocytes according to standard protocols. The FVL and the PT20210A mutations were screened using the primers described previously, with minor modification in the reaction conditions.

Statistical analysis. A logistic regression method was used to calculate the odds ratios (OR) associated with venous thromboembolism. Adjustments for sex and age at the time of the first thrombotic event were made. p values <0.05 were considered to be statistically significant. In the analysis of thrombosis related to contraceptive use or pregnancy, only women of childbearing age were considered. This included all women who, independently of their age at the moment of interview in this study, had experienced their first venous thromboembolism during childbearing age. A Kaplan-Meier analysis was used to estimate the thrombosis-free survival (in years) of the different groups. The reference group was composed of the non-deficient relatives. Differences between factors were analyzed by the log-rank test. The Cox regression method was employed to determine the OR of thrombotic risk with respect to non-deficient individuals, adjusted for sex and type of individual (propositus or relative). The adjustment for type of individual obviated the need to calculate the risks after exclusion of the probands.

We compared the incidence rates of first venous thrombotic events in relatives with (carriers) and without the same deficiency (non-carriers) between different groups of families. We calculated these incidence rates by counting patient-years of observation (follow-up time) and dividing the number of events in each group by the total number of patient-years of all the individuals in the group. Follow-up for symptomatic individuals started at birth and ended at the time of the first venous thrombosis. Follow-up for asymptomatic individuals started at birth and ended at the time of the interview.

Results

Laboratory screening of families
One hundred and thirty-two unrelated families with thrombophilic defects were studied. The FVL mutation was found in propositi with other deficiencies: 2 with AT deficiency (18.2%), 5 with PC deficiency (14.7%) and 4 with PS deficiency (10.0%, 3 type III and 1 type I). The FVL mutation was present in other family members also, but we found relatives with the FVL mutation in families whose probands did not have it. All of the patients in whom the FVL mutation was detected were heterozygous. The PT20210A allele was detected in one family with AT deficiency (9.1%), in 7 with PC deficiency (20.6%) in 5 with PS deficiency (12.5%) and in 6 with the FVL mutation (18.8%). Relatives carrying the PT20210A mutation, but without this defect in the proband, were observed in 19 individuals, two of whom were homozygous. One was a proband who was also heterozygous for the FVL
mutation, and who had suffered from thrombosis at the age of 26 after starting to take contraceptive pills. The other homozygous individual was also a woman without associated defects who belonged to a family with the FVL mutation. She is currently asymptomatic for venous thromboembolism. Cases with three defects were not found.

When we compared the proportion of probands in whom the FVL was associated with some other defect with the prevalence of the FVL mutation in our general population (6/201, 2.9% 95% CI: 1.1-6.4; data not reported), we observed a higher than expected proportion in patients with both defects. This tended to be significant in the case of PC deficiency plus FVL mutation (difference with the general population: 11.7, 95% CI: -0.4-23.9). The same was true for the association of the PT20210A mutation and the prevalence of this mutation in the general population (13/201, 6.5% 95% CI: 3.5-10.8). In the case of PC deficiency plus PT20210A mutation there was a tendency towards the difference in frequency being significant (difference: 14.1, 95% CI: 0.1-28.1). When both mutations were considered together, the prevalences of deficiencies associated with some of them were higher than expected and were significant in the PC and PS deficiencies and almost significant in AT deficiency.

Clinical parameters

The number of thrombotic and asymptomatic individuals with different types of deficiencies is shown in Table 2. The clinical characteristics of the thrombotic patients are also listed in Table 2. The most interesting clinical finding was that women with the PT20210A or the FVL mutation tended to have oral contraceptive-related thrombosis (OCRT). Six out of 11 women with the FVL mutation had OCRT. Moreover, 3 out of 3 with both mutations had OCRT. When women with only one defect and women in whom the PT20210A mutation was associated were considered separately, OCRT was more frequent in the latter group (difference 30.9%, 95% CI 1.2-60.5). The percentages of recurrent thrombosis are shown in Table 2. Despite the lack of differences, there was a tendency towards a higher proportion of multiple thromboses in patients in whom PC deficiency was associated with the FVL or the PT20210A mutation compared with in patients with isolated PC deficiency. Similar results can be seen in the FVL mutation group and the FVL plus PT20210A mutation group. The lowest frequency of recurrent thromboembolism was in isolated carriers of PT20210A (35%).

The incidence rates of first venous thrombosis, studied in each group of families with different...
Defects and after exclusion of cases with other defects, were as follows: in antithrombin deficiency 2.01% per year (p/y) in all cases versus 2.94% p/y without probands (anyone in non-deficiency); in PC deficiency 1.15% p/y versus 0.36% p/y without probands (anyone in non-deficiency); in PS deficiency 1.67% p/y (versus 0.08% p/y in non-deficiency) and when probands were excluded 1.04% p/y (versus 0.08% p/y in non-deficiency); in FVL carriers 1.17% p/y (versus 0.12% p/y in non-carriers) in all cases and when probands were excluded 0.31% p/y (versus 0.12% p/y in non-carriers); in PT20210A carriers 0.8 (versus 0.17% p/y in non-carriers) and 0.23% p/y without probands (versus 0.17% p/y in non-carriers).

Thrombosis risk and association with the FVL or the PT20210A mutation

Compared with non-deficient individuals, the risk of thrombosis increased in patients with AT, PC, and PS deficiencies, in patients with the FVL and PT20210A mutations, and in patients in whom the PT20210A or the FVL mutation was associated. The corresponding odds ratios adjusted for sex and type of individual (propositus or relative) are shown in Table 1. The risk of thrombosis for subjects with the PT20210A mutation was lower than that for other coagulation defects (4.2, 95% CI: 1.8-9.8). The thrombosis-free survival curves for particular defects with or without the PT20210A or the FVL mutation were calculated. The probability of an antithrombin-deficient subject being free of thrombosis was 50% at 34 years (95% CI: 25-43 y.), at 19 years or at 21 years if he or she had the FVL or the PT20210A mutation, respectively (Figure 1). At 62 years, 50% (95% CI: 43-81) of the patients with PC deficiency presented at least one manifestation of venous thrombosis. When the FVL or the PT20210A mutation was associated in these patients, the corresponding ages were 33 years (95% CI: 21-45) and 44 years (95% CI: 16-72), respectively (Figure 2). As for PS deficiency, 50% of the deficient individuals had thrombosis at 37 years (95% CI: 31-43), or at 24 years (95% CI: 0-50) and 36 years (95% CI: 23-49) when the deficiency was associated with the FVL mutation or the PT20210A mutation, respectively (Figure 3). The patients with FVL mutation had a 50% probability of being free of thrombosis at 50 years of age (95% CI: 47-53) or at 52 years (95% CI: 34-70) when the PT20210A mutation was associated (Figure 4). Age at the time of 50% thrombosis-free survival was 65 years for subjects with the PT20210A mutation (95% CI: 57-73) (Figure 5). Comparisons between the thrombosis-free survival curves did not yield statistical differences, probably because of the small number of cases. We observed that the thrombosis-free survival of PC deficient individuals with the FVL mutation tended to be significant (p=0.0882).

Discussion

Biological defects causing thrombophilia are clinically relevant not only to the patient, but also...
to asymptomatic relatives and offspring who may carry the same defect. The type of abnormality determines the mode and duration of treatment and the use of more intense prophylaxis in future thrombotic risk situations. Moreover, thrombosis without environmental triggering factors often occurs in affected individuals.

This study compared the risk of thrombosis in individuals with inherited thrombophilia, due to the FVL or PT20210A mutation, or to AT, PC, or PS deficiencies, when these defects were isolated or appeared in conjunction with the FVL or the PT20210A mutation. In agreement with earlier reports\textsuperscript{10,17} the probability of developing thrombosis during a lifetime was increased 10.6 times for carriers of AT deficiency, 6.4 for PC deficiency and 7.6 for PS deficiency. However, for the FVL mutation (6.2), our findings differ from those of Martinelli et al.\textsuperscript{10} probably because we did not consider arterial thrombosis. Moreover, we found a lower risk of thrombosis in subjects with PT20210A mutation (odds ratio 4.2) than in individuals with other defects. This is the first family study that describes the thrombotic risk of individuals with inherited thrombophilia due to PT20210A mutation and compares it with the risks associated with other genetic defects.

The incidence rate of first venous thrombosis for family member carriers of FVL mutation was 0.31% p/y, similar to the rate recorded by Middeldorp et al.\textsuperscript{18} (0.4% p/y) but different from that reported by Lensen et al.\textsuperscript{11} who found the incidence rate was 0.9% p/y. These comparisons emphasize that the estimated risk of thrombosis for this defect depends on the inclusion criteria in family studies. On the other hand, we found that the incidence rates of first venous thrombosis were very similar for patients with PC deficiency and FVL mutation. A similar conclusion was drawn by Lensen et al.\textsuperscript{11} who studied FVL mutation and compared their data with data from a study performed by Allaart et al.\textsuperscript{19} on similar selected thrombophilic families with heterozygous PC deficiency.

The mean age at the first thrombotic episode ranged from 30 to 40 years for all the patients with single defects. Patients in whom the FVL or the PT20210A mutation and PS or AT deficiencies were associated presented with thrombotic events at a younger age. By contrast, the presence of other defects in patients with PC deficiency or with the FVL mutation did not advance the age of presentation. This is in agreement with recent findings in heterozygous relatives with the FVL mutation or the PC deficiency, in whom the differences in age at the time symptoms developed were attributed to how the patient was selected and not to the type of defect.\textsuperscript{20}

The types of thrombotic manifestation were not statistically different between individuals with different defects. However, superficial thromboses were more frequent in patients with the FVL mutation or with PS deficiency. Moreover, in agreement with recent data, our patients who were FVL mutation carriers tended to have less pulmonary embolism than those with other defects.\textsuperscript{21}
mechanism associated with a lower incidence of pulmonary embolism in carriers of the FVL mutation is unknown.

Our data agree with the reported relationship between oral contraceptive intake and venous thrombosis in FVL and PT20210A carriers. Based on these findings and earlier data, screening for both mutations should be performed in women who wish to take oral contraceptives if they belong to a thrombophilic family.

We found that the prevalences of the FVL mutation in families with other deficiencies (18.2% in families with AT deficiency, 14.7%, in families with PC deficiency and 10.0% in families PS deficiency) were higher than expected for our general population. These findings agree with those described by other authors in families with AT, PC and PS deficiencies. As for the PT20210A mutation, a similar situation was observed: our general population has the highest prevalence ever reported (6.5%).

As for the PT20210A mutation, a similar situation was observed: our general population has the highest prevalence ever reported (6.5%). And in the families with thrombophilic defects the prevalence of the PT20210A mutation was also higher than expected. PT20210-FVL was the most common combined defect in these families. The literature on this point is contradictory. Ehrenforth et al. and Howard et al. both reported a higher prevalence of the PT20210A mutation in heterozygotes for the FVL mutation (12.2% and 10.4%, respectively). By contrast, other authors, Cumming et al. and Hillarp et al. reported that this association was infrequent. A number of factors could account for these apparently contradictory data. First, the low prevalences in the general population and in the thrombotic popu-
Genetic risk factors for thrombosis in thrombophilic families

Cohort On Thrombophilia-EPCOT) addressing this question. The EPCOT study seeks to determine the risk of thrombosis in carriers of prothrombotic deficiencies and to establish whether long-term thromboprophylaxis should be recommended.

It is well known that the co-existence of PT20210A and FVL mutations tends to raise the risk of recurrent thrombosis. The risks of thrombosis for particular deficiencies are probably impossible to determine given that a number of unknown hereditary factors remain to be identified. These factors may be protective, but they may also be deleterious. For this reason, the risk of thrombosis of a particular individual could vary when other risk factors are discovered. At present, clinical assessment continues to play an important role in the evaluation of affected individuals.

Contributions and Acknowledgments

IT performed the laboratory tests, wrote the paper and was involved in the design of the study, selection of patients, analysis and interpretation of data. JM and JMS wrote part of the paper and were involved in the analysis and interpretation of data. AO was involved in the statistical analyses and interpretation of data. JCS helped to select patients. IC and EM-S performed some of the genetic analyses. MB was in charge of the plasma analyses. CV and IT performed the laboratory tests, wrote the paper and was involved in the design of the study, selection of patients, analysis and interpretation of data. AO was involved in the statistical analyses and interpretation of data. JCS helped to select patients. IC and EM-S performed some of the genetic analyses. MB was in charge of the plasma analyses. CV

Potential implications for clinical practice

The high prevalence of the FVL and PT20210A mutations in the Spanish population warrant screening for both mutations in thrombotic patients, especially in patients exhibiting additional thrombophilic defects. Such screening should ultimately lead to a reduction in thromboembolic morbidity and mortality.

References

9. Bovill EG, Hasstedt SJ, Leppert MF, Long GL. Hereditary thrombophilia as a model for multigenic disorder: The high prevalence of the FVL and PT20210A mutations in the Spanish population warrant screening for both mutations in thrombotic patients, especially in patients exhibiting additional thrombophilic defects. Such screening should ultimately lead to a reduction in thromboembolic morbidity and mortality.

Disclosures
Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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