



Factor V Leiden increases plasma F1+2 levels both in normal and deep venous thrombosis subjects

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ABSTRACT

Background and Objectives. A simple approach to understanding molecular mechanisms leading to thrombosis is the definition of how genetic factors influence biochemical parameters of coagulation. Conflicting data have been reported regarding the role that the genotype of factor V plays in the control of plasma F1+2 levels. The aim of this study was to test whether the factor V Leiden mutation affects F1+2 levels.

Design and Methods. We studied the effect of factor V Leiden mutation (detected by the polymerase chain reaction technique) on plasma F1+2 levels in 418 normal subjects and 39 subjects affected by deep venous thrombosis.

Results. In both normal subjects and those with venous thrombosis, heterozygotes for the Leiden mutation showed significantly higher plasma levels of F1+2 ($p < 0.0001$ and $p < 0.005$, respectively). Subjects with venous thrombosis had a higher allelic frequency of the Leiden mutation than normal subjects (11.5% and 3.1%, respectively).

Interpretation and Conclusions. The results indicate that the genotype of factor V is a determinant of plasma F1+2 concentration. The allelic frequency of Leiden mutation in our normal subjects is higher than that reported for populations of north- and middle-Europe. This finding is consistent with the peculiar ancestry and history of Friuli (the area in which subjects for this study were recruited), with respect to other Italian regions.

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Key words: factor V, Leiden, F1+2, multifactorial trait, thrombosis

In order to understand mechanisms leading to thrombosis, it is critical to define how genetic factors influence coagulation. A simple approach to this end is the study of the relationship existing between genetic factors and biochemical parameters of coagulation. The plasma concentration of the prothrombin activation fragment 1+2 (F1+2) is an index of thrombin generation, high values indicating a hypercoagulable state.¹ Plasma F1+2 levels are modified by environmental factors such as pregnancy,² cig-

arette smoking,³ oral contraceptive treatment,⁴ and anticoagulation therapy.⁵ Genetic disturbances including hereditary protein C and protein S deficiency have also been identified as modifiers of F1+2 levels.⁶⁻⁸ The multiplicity of both environmental and genetic factors that control F1+2 levels indicates that this parameter could represent a typical multifactorial trait.

A variant of the prothrombin gene, the G to A transition at nucleotide 20210 (G20210A), in heterozygous subjects is a risk factor for deep venous thrombosis (DVT) and is associated with increased prothrombin activity.⁹ However, subjects heterozygous for this variant show no difference in F1+2 levels with respect to subjects not carrying the G20210A mutation.¹⁰ Congenital antithrombin III deficiency does not significantly modify plasma F1+2 levels.^{8,11} Thus, in subjects with protein C or protein S deficiency, hypercoagulability is due to ongoing thrombin generation. In contrast, hypercoagulability in subjects with the prothrombin G20210A allele or with congenital antithrombin deficiency is due to increased potential to generate thrombin once coagulation is activated.¹²

A single point mutation of the gene coding for coagulation factor V, in which adenine replace guanine at nucleotide 1691 (G1691A), results in a form of factor Va, called factor V Leiden (FV Leiden), which is resistant to degradation by activated protein C.¹³ This genetic variant is associated with an increased risk of DVT.¹⁴ The high allelic frequency of FV Leiden in Caucasians (1.5% to 4%, depending on the populations),¹⁵ has meant that this genetic polymorphism is emerging as very important in defining the genetic risk of DVT. Conflicting data have been reported on the role that FV Leiden may have on the plasma concentration of F1+2.^{7,16-19} In this study we addressed the question of whether F1+2 levels are modified by the presence of FV Leiden in both normal subjects and in subjects with DVT.

Design and Methods

Subjects

The normal control subjects were a cohort of 418 apparently healthy adults aged between 20 and 55 years old enrolled at Udine University Hospital. The DVT group consisted of 39 patients aged between 25 and 61 years who had had at least one well documented DVT episode. DVT was diagnosed from clinical data and documented by Doppler ultrasonography or contrast venography in each patient. Pulmonary embolism (4 cases) and spontaneous fetal

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loss (2 cases) had also been documented in some of the DVT patients. At the time of blood collection DVT subjects were free from thrombotic manifestations. Individuals included in the study were not receiving anticoagulant or antiaggregant therapy at the time of blood collection. Subjects were excluded if they tested positive for a lupus anticoagulant or demonstrated APTT values abnormally prolonged relative to our coagulation laboratory's normal reference range.

Detection of the FVR506Q mutation of the factor V gene

Blood samples were collected in trisodium citrate, and genomic DNA was prepared by standard procedures. A 206-base pairs (bp) DNA fragment of the factor V gene that includes nucleotide 1691 was amplified by polymerase chain reaction (PCR) with the forward primer 5' CATACTACAGTGACGTGGAC 3' and the reverse primer 5' TGTTCT CTTGAAG-GAAATGC 3'. The PCR was carried out in 50 μ L of reaction mixture consisting of 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂) (Perkin Elmer), 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μ M of each deoxynucleotide triphosphate, 50 pmol of each primer, 200 ng of genomic DNA and 1.25 U of Taq polymerase (AmpliTaq, Perkin Elmer). The PCR was performed in 33 cycles consisting of 30 seconds at 94°C, 30 seconds at 63°C, 30 seconds at 72°C and a 7-minute elongation final at 72°C. At the end of PCR amplification, 30 μ L were digested with 5U of *Mnl* I (New England Biolabs) by incubation at 37°C for 3 hours to overnight. Digested PCR products were separated by 10% polyacrylamide gels in 0.5 X TBE and silver stained (Pharmacia). Digestion of the wild-type allele with *Mnl* I generates three fragments 123, 47 and 36 bp, whereas digestion of the FVR506Q allele results in the generation of two fragments of 159 and 47 bp.

Determination of F1+2 by enzyme immunoassay

For the determination of F1+2, nine volumes of venous blood were mixed with one volume of 0.129 mol/L trisodium citrate and centrifuged at 2,500 rpm for 15 minutes at room temperature. F1+2 was assayed using a commercially available ELISA test (Enzygnost F1+2 micro, Dade Behring). This test is based on the sandwich procedure, with a first rabbit polyclonal antibody against the carboxyterminal end of F1+2 fixed to the surface of the microtitration plate by which the F1+2 molecules present in the sample are captured and a second peroxidase-conjugated antibody by which the number of F1+2 molecules bound to the microtiter plate is revealed.

Results

Plasma levels of F1+2 were measured in our cohort of normal subjects. Results are shown, as frequency distribution,²⁰ in Figure 1. A positively skewed distribution of F1+2 values was observed. In particular, descriptive analysis of the data shows a F1+2 median value of 0.68 nmol/L (range: 0.30-1.97; 25th-75th percentiles: 0.53-0.95). Plasma F1+2 levels in normal subjects and DVT patients were compared by the non-parametric Mann-Whitney test. Significantly higher F1+2 levels ($p < 0.0001$) were observed in DVT patients (median value: 1.05 nmol/L; range: 0.32-

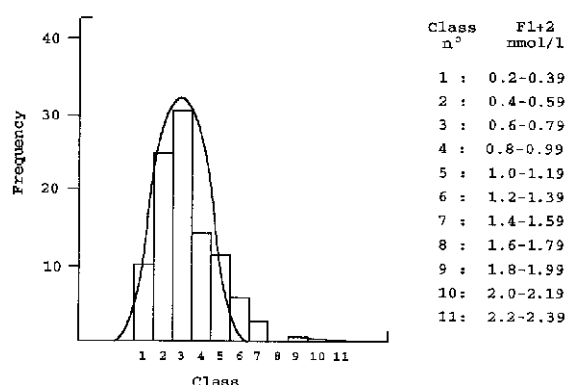


Figure 1. F1+2 levels in normal subjects. Each bar of the graph represents the class of F1+2 values shown on the right. The frequency distribution of F1+2 levels of normal subjects is positively skewed with respect to a curve with normal distribution.

Table 1. Distribution of the transition G to A at position 1691 of factor V gene (FV Leiden) among normal and DVT subjects.

Genotype	Normal subjects (n=418)	DVT patients (n=39)
G/G	94.02% (n=393)	77% (n=30)
G/A	5.70% (n=24)	23% (n=9)
A/A	0.2% (n=1)	

G/G and A/A indicate subjects homozygous for the normal and Leiden mutation of the factor V gene, respectively. G/A indicates heterozygous subjects.

2.73; 25th-75th percentiles: 0.76-1.81) than in normal subjects (see above).

The presence of FV Leiden was determined in both normal and DVT subjects. Results are presented in Table 1. In the control group, 1 homozygous and 24 heterozygous subjects were found; the allelic frequency was, therefore, 3.1%. This value is higher than other reported values for Italian populations.^{15,21-23} In the DVT group, 9 out of 39 patients were heterozygous for FV Leiden, the allelic frequency, therefore, being 11.5%. The difference in FV Leiden allelic frequencies between normal and DVT subjects was highly significant ($p=0.0011$, odds ratio 4.15, 95% confidence interval 1.44 to 10.60). These results confirm that FV Leiden is a risk factor for DVT.

In Figure 2 the distribution of F1+2 levels in both control and DVT subjects with respect to the factor V genotype is shown. In both groups, subjects carrying the FV Leiden (genotype G/A in the figure) had a different distribution of F1+2 levels than those not harboring the mutation. While in control subjects clear peaks in the frequency of F1+2 levels distribution can be observed (class 3 for G/G subjects; class 4 for G/A subjects), in DVT subjects the distribution of the frequency was much smoother. Although caution should be used to interpret these results because of the small sample size, this finding seems to suggest that, irre-

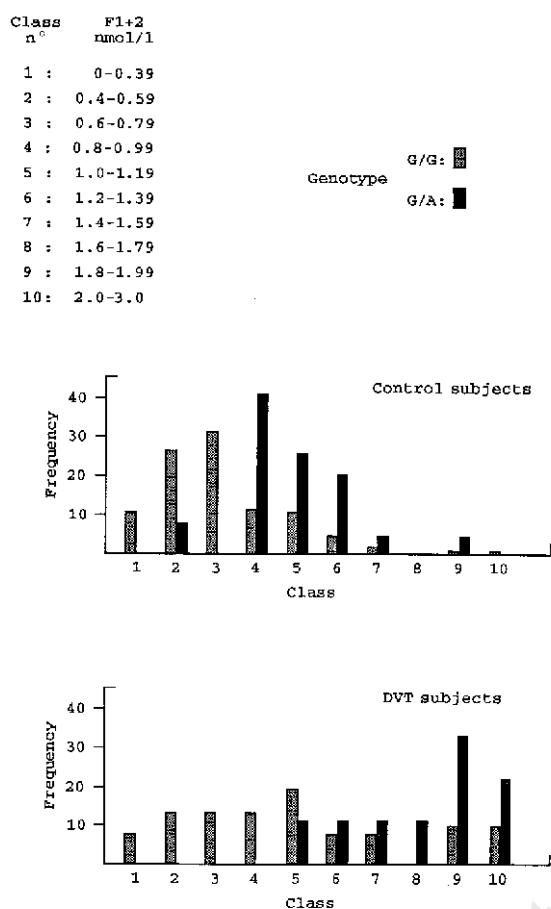


Figure 2. Factor V Leiden increases F1+2 levels in both normal and DVT subjects. Each bar of the graph represents the class of F1+2 values shown on the top left of the figure. Grey bars indicate classes of subjects with the normal factor V genotype; black bars indicate classes of subjects carrying the Leiden mutation.

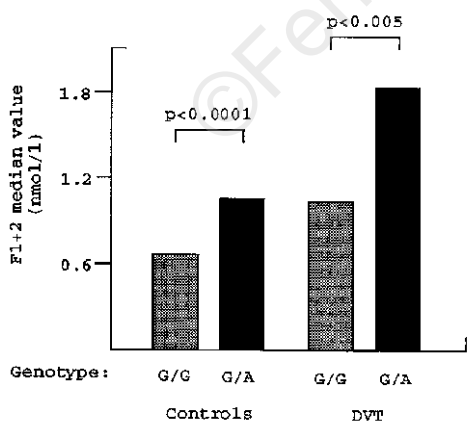


Figure 3. Median values of F1+2 levels are significantly different in both normal and DVT subjects, depending on the presence of factor V Leiden. Grey bars indicate median F1+2 values in subjects with the normal factor V genotype; black bars indicate F1+2 median values of subjects carrying the Leiden mutation. Significance was calculated using the non-parametric Mann-Whitney test.

spectively of the factor V genotype, venous thrombosis is a heterogeneous entity in terms of plasma F1+2 concentration and, therefore, that the value of F1+2 levels as a clinical predictor of DVT is limited.

The median values of F1+2 levels in control and DVT subjects, both grouped according to the factor V genotype, are shown in Figure 3. In both groups the presence of FV Leiden (genotype G/A in the figure) was associated with significantly higher plasma F1+2 levels. Taken together these findings indicate that factor V genotype is a determinant of plasma F1+2 concentration in both normal and DVT subjects.

Discussion

Conflicting data have been reported regarding the role that the genotype of factor V plays in the control of plasma F1+2 levels. Lambropoulos *et al.*¹⁶ studied patients with an unexplained thrombophilic tendency and found no significant difference in levels of F1+2 between subjects with different factor V genotypes. Kirlé *et al.*¹⁷ reported that F1+2 levels in patients with thrombosis were higher than in normal subjects; however, no difference was observed between patients with or without FV Leiden. In a cohort of acute stroke cases, F1+2 levels were higher than in normal controls, but not related to the factor V genotype.¹⁸ Dahlback's group studied the relationship between F1+2 levels and factor V genotype in members of thrombosis-prone families.⁷ It was found that the plasma levels of F1+2 were higher in subjects harboring the FV Leiden than in non-carrier relatives. Simioni *et al.*¹⁹ compared the plasma F1+2 levels of FV Leiden carriers with those of non-carrier relatives. Establishing F1+2 cut-off values as the higher limits of normal ranges in healthy subjects it was found that 68.8% of FV Leiden carriers exhibited F1+2 levels above the cut-off, while only 38.9% of non-carrier relatives had F1+2 levels above the cut-off. However, in this last study, median F1+2 levels in carriers of FV Leiden were not found to be significantly higher than those found in non-carriers. Our data clearly demonstrate that the presence of FV Leiden increases plasma F1+2 levels both in normal and DVT subjects and are in good agreement with those reported by Dahlback's group.⁷ A major reason explaining why only the present investigation and those of Dahlback's group, but not other studies, have demonstrated a clear role for factor V genotype in controlling plasma F1+2 concentration could be the relatively low variability of F1+2 levels recorded in our study and that by Dahlback's group. It is not clear whether the different variabilities of F1+2 levels are due to technical reasons or to population differences.

In our normal subjects we found a FV allelic frequency of 3.1%. This is higher than other reported values for Italian populations,^{15,21-23} which, at most, reached half our value. However, the allelic frequency of FV Leiden in our population is similar to that reported for populations of north- and middle-Europe.^{15, 24-26} This finding could be due both to the Celtic ancestry of Friuli's population and to the high frequency of contacts established with middle-Europe populations over the past twenty centuries.

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FM contributed to the design of the study and did most of the DNA assays. RG contributed to the analysis of the data and recruiting normal subjects. FP contributed to the analysis of the data and to write the paper. RC did most of the biochemical assays. DF took part in the DNA assays as well as in interpretation of results. MGM recruited and characterized DVT patients. AC took part in interpreting results and writing the paper. GD formulated the design of the study, contributed to the analysis of the data and wrote the paper. The first author contributed to design of the study and did most of DNA assays. The last author formulated the design of the study, contributed to the analysis of the data and wrote the paper. Each of the other co-authors contributed to several aspects of this work but in a less relevant manner than the first and the last authors.

Disclosures

Conflict of interest: none.

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Potential implications for clinical practice

- ◆ Carriers of the Leiden mutation have a three to four fold higher risk of developing DVT.
- ◆ High plasma F1+2 levels may represent an indication for genetic testing for the Leiden mutation.

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