New Prothrombin Mutation (Arg596Trp, Prothrombin Padua 2) Associated With Venous Thromboembolism

Cristiana Bulato, Claudia Maria Radu, Elena Campello, Sabrina Gavasso, Luca Spiezia, Daniela Tormene, Paolo Simioni

**Objective**—Two different prothrombin variants, p.Arg596Leu and p.Arg596Gln, conferring antithrombin resistance to patients with venous thromboembolism have been recently reported. Here, we describe a novel substitution affecting Arg596 of prothrombin molecule (Arginine596 to Tryptophan or p.Arg596Trp or Arg221aTrp in the chymotrypsinogen numbering system or prothrombin Padua 2) in 2 Italian families with venous thromboembolism.

**Approach and Results**—Prothrombin Padua 2 has been characterized either in plasma of carriers or using Arg596Trp recombinant prothrombin. Routine coagulation tests, thrombin generation, and antithrombin resistance tests were performed, as well as measurement of the levels of thrombin–antithrombin complexes. All carriers were heterozygotes and presented with a mild reduction of the prothrombin activity. Thrombin generation in carriers showed only a markedly prolonged decay. This finding was confirmed in plasma reconstituted with Arg596Trp recombinant prothrombin mimicking a homozygous condition, which showed longer decay and higher endogenous thrombin potential in thrombin generation than wild-type recombinant prothrombin reconstituted plasma. Patient’s plasma as well as Arg596Trp recombinant prothrombin showed a clear thrombin resistance to antithrombin inactivation. These findings were supported by the assessment of thrombin–antithrombin complexes formation, which was strongly reduced for Arg596Trp recombinant prothrombin as compared with wild-type recombinant prothrombin. In a series of 400 unrelated consecutive patients with venous thromboembolism, 2 carriers of prothrombin Padua 2 were found (estimated prevalence of 0.5%).

**Conclusions**—Our study showed that prothrombin Padua 2 induces antithrombin resistance and is associated with an increased risk of venous thromboembolism. Codon 596 (CGG) of prothrombin is a hot spot for mutations, which constitute a new and relatively frequent cause of inherited thrombophilia. (Arterioscler Thromb Vasc Biol. 2016;36:1022-1029. DOI: 10.1161/ATVBHA.115.306914.)

**Key Words:** antithrombin ■ mutation ■ prothrombin ■ thrombophilia ■ venous thromboembolism

Numerous gene mutations in various molecules have been found in families with inherited thrombophilia, but many mutations remain unidentified.1 Prothrombin, the precursor of thrombin, is a serine protease that plays a key role in hemostasis and thrombosis. The prothrombin G20210A is a risk factor for venous thromboembolism (VTE).2 Recently, the prothrombin Yukuhashi mutation (c.1787G>T, p.Arg596Leu) was identified in a Japanese family.3 The mutant recombinant prothrombin showed moderately lower clotting activity than the wild type but a substantial resistance to the inhibitory effect of antithrombin, which resulted in an increased risk of thrombosis in affected family members. Another mutation was found in prothrombin Belgrade, prothrombin Amrta, and, recently, in a Japanese family, characterized by an Arginine to Glutamine substitution at the same position of the prothrombin molecule (c.1787G>A, p.Arg596Gln).4,4 Here, we studied a novel gain-of-function F2 mutation affecting the same amino acid residue, c.1786C>T (p.Arg596Trp; Arg221aTrp in the chymotrypsinogen numbering system5), named prothrombin Padua 2 by the name of the City it was discovered, inducing antithrombin resistance in 2 probands and several affected members from 2 Italian thrombophilic families.

**Case Report**

The proband from Family 1 is a 47-year-old white man from North East of Italy who was admitted in January 2014 to our Hospital with objectively documented unprovoked occlusive right leg femoral–popliteal deep vein thrombosis (DVT) and pulmonary embolism. He had experienced the first episode of superficial thrombophlebitis to the right saphena magna vein at the age of 38 and subsequent recurrences. Thrombophlebitis episodes were successfully treated with low–molecular weight heparin for 30 to 45 days. Screening for thrombophilia

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and warfarin (international normalized ratio 2.0–3.0). She had been investigated for common thrombophilic conditions before estroprogestinics administration, and no known defect had been investigated for common thrombophilic conditions. Compression ultrasonography showed femoral-popliteal consistency with a diagnosis of massive pulmonary embolism. Angio–computed tomographic scan showed numerous endo–vascular fillings defects of upper and lower lobes of both lungs, consistent with a diagnosis of massive thromboembolism. She had stopped warfarin in January 2014, and no recurrent thrombosis has occurred until now. No family members experienced thrombotic episodes. We are aware of the presence of severe Hemophilia A in several members of the family on the maternal side (where the prothrombin defect comes from). Unfortunately, this part of the family was not available for study. Extensive thrombophilia screening was repeated in the proband before starting warfarin, during the acute phase of VTE. A slightly reduced prothrombin activity (65.5%) in a PT-based clotting assay was detected.

Materials and Methods

Plasma samples from both probands and family members examined (Figure 1A and 1B) showed normal PT, activated partial thromboplastin time (Table 1), and factor VIII, IX, X, and XI activities. Antigen and activity levels of protein C and protein S were within normal limits, as well as fibrinogen levels and antithrombin and plasminogen activities. Factor V Leiden and prothrombin G20210A polymorphisms were not detected in family members. Tests for lupus anticoagulant, anticardiolipin, and anti-beta2-glycoprotein I antibodies were negative.

Prothrombin Activity and Antigen in Family Members

In Family 1, the proband (III-2), his mother (II-2), and sister (III-3) were found to have reduced PT-based prothrombin functional activity (FII:Act), whereas the maternal cousin (III-7) exhibited a PT-based FII:Act slightly below the normal range (Table 1). Prothrombin antigen (FII:Ag) level in the proband was normal, whereas in the mother, sister, and maternal cousin, FII:Ag levels were slightly reduced (Table 1). Three out of 4 affected family members presented with a mild reduction of ecarin clotting time (ECT)–based FII:Act (Table 1). Mean values of FII:Ag and PT-based and ECT-based FII:Act in affected family members were 70.5%, 61.3%, and 70.7% of normal, respectively. The proband’s daughter (IV-1, nonaffected) presented with FII:Ag and FII:Act levels within the normal values. The proband’s mother and maternal cousin have experienced DVT and pulmonary embolism.

In Family 2, the proband (III-1), her sister (III-2), and mother (II-2) showed slightly reduced or borderline PT-based FII:Act with normal FII:Ag levels (Table 1). Two out of 3 affected family members presented with a mild reduction of ecarin clotting time (ECT)–based FII:Act (Table 1). Mean values of FII:Ag and PT-based and ECT-based FII:Act in affected family members were 106.3%, 70.8%, and 69.0% of normal, respectively. The proband’s father (II-1, nonaffected) had normal FII:Ag and FII:Act levels.

F2 Gene Analysis

Data from direct DNA sequencing revealed that both probands were heterozygous for a novel missense mutation in exon 14 of F2, c.1786C>T, resulting in the substitution of an arginine to tryptophan.

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tbody>
<tr>
<td>Arg596Trp rFII</td>
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<tr>
<td>DVT</td>
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<tr>
<td>ECT</td>
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<tr>
<td>ETP</td>
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<tr>
<td>FII:Act</td>
</tr>
<tr>
<td>FII:Ag</td>
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<tr>
<td>PC</td>
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<tr>
<td>PT</td>
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</tr>
<tr>
<td>TG</td>
</tr>
<tr>
<td>VTE</td>
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<tr>
<td>WT rFII</td>
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with tryptophan at residue 596 (p.Arg596Trp or Arg221aTrp in the chymotrypsinogen numbering system) in the prothrombin molecule (Figure 2A).

Sequencing of exon 14 was also performed on all available relatives and demonstrated that in Family 1, the proband’s mother (II-2), sister (III-3), and maternal cousin (III-7) were heterozygous for the p.Arg596Trp mutation. In the proband’s daughter, the mutation was absent.

In Family 2, the same mutation was present in heterozygous form in the proband’s mother (II-2) and sister (III-2). The proband’s father (II-1) had no mutation.

Sequencing of exon 14 in 100 healthy subjects and 100 patients with documented DVT failed to detect the c.1786C>T mutation. In a different series of 400 unrelated consecutive patients with provoked and unprovoked VTE, we found 2 carriers of prothrombin Padua 2 (prevalence of 0.5%).

Table 1. Main Clotting Features of Members of the 2 Families Investigated

<table>
<thead>
<tr>
<th>Family Member</th>
<th>Sex</th>
<th>Age, y</th>
<th>PT, %†</th>
<th>aPTT, s‡</th>
<th>FII:Ag, %§</th>
<th>FII:Act, %§</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Family 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-2, proband</td>
<td>M</td>
<td>47</td>
<td>75.5</td>
<td>28.5</td>
<td>80.0</td>
<td>54.0</td>
<td>67.7</td>
</tr>
<tr>
<td>II-2, mother</td>
<td>F</td>
<td>79</td>
<td>91.3</td>
<td>29.6</td>
<td>60.0</td>
<td>58.0</td>
<td>68.4</td>
</tr>
<tr>
<td>III-3, sister</td>
<td>F</td>
<td>53</td>
<td>78.6</td>
<td>32.9</td>
<td>72.0</td>
<td>58.5</td>
<td>66.3</td>
</tr>
<tr>
<td>III-7, cousin</td>
<td>F</td>
<td>55</td>
<td>86.0</td>
<td>31.0</td>
<td>70.0</td>
<td>74.6</td>
<td>80.4</td>
</tr>
<tr>
<td>IV-1, daughter</td>
<td>F</td>
<td>11</td>
<td>84.3</td>
<td>29.7</td>
<td>92.0</td>
<td>99.5</td>
<td>78.7</td>
</tr>
<tr>
<td><strong>Family 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-1, proband</td>
<td>F</td>
<td>29</td>
<td>77.0</td>
<td>28.7</td>
<td>89.0</td>
<td>65.5</td>
<td>63.1</td>
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<tr>
<td>II-1, father</td>
<td>M</td>
<td>62</td>
<td>107.5</td>
<td>25.2</td>
<td>103.0</td>
<td>117.4</td>
<td>153.8</td>
</tr>
<tr>
<td>II-2, mother</td>
<td>F</td>
<td>58</td>
<td>85.2</td>
<td>30.2</td>
<td>110.0</td>
<td>78.7</td>
<td>83.3</td>
</tr>
<tr>
<td>III-2, sister</td>
<td>F</td>
<td>33</td>
<td>84.8</td>
<td>28.1</td>
<td>120.0</td>
<td>68.2</td>
<td>60.7</td>
</tr>
</tbody>
</table>

aPTT indicates activated partial thromboplastin time; ECT, ecarin clotting time; FII:Act, prothrombin functional activity; FII:Ag, prothrombin antigen; and PT, prothrombin time.

*PT, FII:Ag, and FII:Act are expressed as the percentage of the normal level.
†The normal range for PT is 70% to 100%.
‡The normal range for aPTT is 24.4–36.5 s.
§The normal range for FII:Ag and FII:Act is 80% to 120%.
Restriction Enzyme Analysis
Digestion of a normal PCR-amplified exon 14 with HpaII resulted in DNA fragments of 280 and 187 bp. Because the p.Arg596Trp mutation abolished the HpaII restriction site in exon 14, heterozygous patients showed a 467 bp band together with the 280 and 187 bp bands (Figure 2B).

Recombinant Prothrombin Padua 2 Expression
SDS-PAGE gel and silver staining of purified recombinant prothrombins showed a major band with an apparent molecular weight of 72 kDa, which had a similar mobility to that of normal prothrombin from human plasma (data not shown).

Prothrombin activities of wild-type recombinant prothrombin (WT rFII) and Arg596Trp recombinant prothrombin (Arg596Trp rFII), assessed in reconstituted plasma (final concentration 90 μg/mL; see Materials and Methods in the online-only Data Supplement) using both PT-based and ECT-based assays, are reported in Table 2. Both methods showed an activity of Arg596Trp rFII ≈8× lower than WT rFII (specific activity of Arg596Trp rFII as compared with WT rFII, 12.0% and 12.6% with the PT-based and the ECT-based activity assay, respectively).

Thrombin Generation Assay
Patients’ Plasma
Thrombin generation (TG) curves obtained from plasma samples with 5 pmol/L tissue factor (TF) concentration showed a significant prolongation of the decay in all subjects affected by the mutation as compared with healthy family members and pooled normal plasma. Healthy subjects in Family 1 (IV-1) and 2 (II-1) presented a decay of 18.3 and 23.7 minutes, respectively. These values were similar to that detected for pooled normal plasma (18.7 minutes). Members of the 2 families in which the mutation has been identified showed values of decay from 33.0 to 40.0 minutes. No significant difference was found for the other TG parameters (Figure 3A and 3B).

TG curves obtained from plasma samples with 1 pmol/L TF concentration showed a prolongation of the decay as seen for 5 pmol/L TF concentration. In addition, at 1 pmol/L TF, 6 out of 7 heterozygous carriers presented with a lower peak height, and all carriers showed prolonged lag time as compared with pooled normal plasma (data not shown). Finally, no significant increase of endogenous thrombin potential (ETP) values was seen in heterozygous carriers of the mutation as compared with pooled normal plasma at 1 pmol/L TF (data not shown).

Recombinant Prothrombins
TG was measured in WT rFII or Arg596Trp rFII reconstituted plasma by triggering coagulation with 5 pmol/L TF. Arg596Trp rFII-reconstituted plasma showed reduced peak, prolonged lag time and decay, and increased ETP in comparison to WT rFII reconstituted plasma. The heterozygous condition, obtained by reconstituting prothrombin-deficient plasma with 50% WT rFII and 50% Arg596Trp rFII, exhibited values of lag time, ETP, peak, and decay that were intermediate between those obtained for the homozygous WT rFII and the homozygous Arg596Trp rFII (Figure 4).

Antithrombin Resistance Analysis
Patients’ Plasma
Test for detection of antithrombin resistance was performed on plasma samples from all prothrombin Padua 2 mutation carriers, including those asymptomatic, and unaffected relatives of both families, in the absence and presence of heparin.
As regards Family 1, after 30 minutes of inactivation using antithrombin without heparin, the only unaffected available family member (the proband’s daughter, IV-1) showed a relative residual thrombin activity (RRTA) of 29.0 ± 0.1%, whereas all mutation carriers investigated had values from 34.7 ± 0.1% to 42.7 ± 1.1% (III-2, II-2, III-3 and III-7) (Figure 5A).

In Family 2, the proband’s father (II-1), a noncarrier for the mutation, showed an RRTA of 23.8 ± 0.2%, whereas the proband (III-1) and her affected relatives studied (II-2 and III-2) had values from 36.1 ± 0.1% to 45.1 ± 0.9% (Figure 5B). The analysis performed in the absence of heparin did not reveal a significant difference in terms of RRTA between subjects with and without prothrombin Padua 2 mutation. On the contrary, after 1 minute of inactivation using antithrombin with heparin, subjects with prothrombin Padua 2 mutation of both families showed values of RRTA significantly higher (≈ 90%) than those obtained from subjects without the mutation (≈ 50%). This difference further increased after 5 minutes incubation of plasma samples with antithrombin and heparin (Figure 5C and 5D).

Recombinant Prothrombins

Data reported in Figure 6A indicate that homozygous Arg596Trp rFII was not inhibited even after 30 minutes incubation with a large excess of antithrombin (100 mU/mL), whereas WT rFII showed an RRTA of 17.4 ± 0.1%. Heterozygous Arg596Trp rFII showed higher RRTA (73.0 ± 0.4%) as compared with WT rFII but lower than homozygous Arg596Trp rFII (94.9 ± 1.5%).

Test for detection of antithrombin resistance was performed also in the presence of 5 U/mL of heparin. As shown in Figure 6B, after 2 minutes inactivation using antithrombin plus heparin, RRTA of WT rFII, heterozygous Arg596Trp rFII, and homozygous Arg596Trp rFII were 18.6 ± 0.3%, 44.0 ± 0.4%, and 86.5 ± 0.8%, respectively.

Formation of Thrombin–Antithrombin Complexes

Patients’ Plasma

Thrombin–antithrombin complexes levels, measured in plasma of subjects heterozygous for the mutation, were within the normal range (data not shown).
Recombinant Prothrombins

In the absence of heparin, thrombin–antithrombin complexes concentration between WT rFII and antithrombin increased over time and reached a plateau after 30 minutes of incubation, whereas in the same incubation period, Arg596Trp rFII showed a slight increase of binding with antithrombin. The presence of heparin accelerated the formation of complexes with antithrombin for both recombinant proteins, but the concentration of complexes, measured for 5 minutes, were much lower for Arg596Trp rFII (Figure 7A and 7B).

Cleavage of Fibrinogen by Recombinant (Pro)thrombins With a Clotting Method

Clotting times for WT rFII, heterozygous, and homozygous Arg596Trp rFII were 18.6±0.5, 34.2±1.1, and 210.2±56.5 sec,

Figure 5. Antithrombin (AT) resistance analysis in plasma samples. Test for detection of AT resistance was performed in all members of the 2 families investigated in the absence and presence of 1 U/mL heparin. In Family 1, III-2, II-2, III-3, and III-7 are prothrombin Padua 2 mutation carriers, whereas IV-1 is a healthy relative. In Family 2, III-1, II-2, and III-2 are members affected by the mutation, whereas II-1 is a healthy relative. Plasma prothrombin, once converted into thrombin by 110 mU/mL ecarin, was inactivated using 50 mU/mL AT without heparin for 30 minutes (A and B) and with heparin for 5 minutes (C and D). Relative residual thrombin activities (RRTA) were calculated by comparison with data obtained at 0 minutes and expressed as percentage. Measurements were done in triplicate and expressed as mean±SD.

Figure 6. Antithrombin (AT) resistance assay for the recombinant prothrombins. Recombinant prothrombins were converted into thrombins by 50 mU/mL ecarin and then inactivated with 100 mU/mL AT with and without 5 U/mL heparin. Inactivation reactions were followed for 30 minutes in the absence of heparin (A) and for 5 minutes in the presence of heparin (B). Relative residual thrombin activities (RRTA) were calculated as described in Figure 5. Measurements were performed in triplicate and reported as mean±SD.
Discussion

A new missense mutation (c.1786C>T) at the last exon of F2, which results in an Arginine to Tryptophan substitution at residue 596 (p.Arg596Trp or Arg221aTrp in the chymotrypsinogen numbering system or prothrombin Padua 2), has been found in 2 unrelated Italian families. We assumed that prothrombin Padua 2 behaved similarly to the 2 prothrombin variants with amino acid substitutions at the same position recently described.3–6 Here, we characterized prothrombin Padua 2 either in patients’ plasma or after expression of the recombinant molecule.

PT and activated partial thromboplastin time were normal in all family members. In addition, not all affected members investigated showed reduced FII:Act using a PT-based clotting assay, some of them presenting with activity values at the lower limit of the normal range. This fact has clinical implication because the presence of prothrombin Padua 2 (and possibly other similar prothrombin variants) cannot be suspected on the basis of a prolongation of global tests routinely performed. However, in the presence of normal PT and activated partial thromboplastin time, isolated reduction of functional prothrombin activity in plasma and impaired fibrinogen cleavage may prompt further investigation to exclude these prothrombin mutants. As for other global tests such as standard TG in plasma with 5 pmol/L TF, it has to be noted that all parameters were normal in carriers, except for decay values. All carriers of both mutations at Arg596 showed a clear antithrombin resistance, particularly during the first 5 minutes of the inactivation reaction.

Collectively, these experiments suggest that prothrombin Padua 2 can cause antithrombin resistance as previously shown for other similar mutants.3–6 Some authors3,8 have emphasized the importance of Arg596 (Arg221a), located in the sodium-binding site of thrombin, in stabilizing the interaction between thrombin and antithrombin. In particular, the side chain of Arg596 (Arg221a) in thrombin forms 2 hydrogen bonds with the side chain of Asn265 in antithrombin. The exact mechanisms by which these new prothrombin variants, including prothrombin Padua 2, cause thrombotic manifestations are not fully elucidated. It is peculiar that in the presence of heparin, RRTA measured in plasma of probands and their affected relatives after addition of antithrombin were only slightly higher than in members without the mutation. In contrast, in the presence of heparin, all carriers of the mutation showed a clear antithrombin resistance, particularly during the first 5 minutes of the inactivation reaction.

How frequent are these new F2 mutations and particularly prothrombin Padua 2? With a quite selective and arbitrary fact, the final clinical effect is unpredictable being hypercoagulable on the basis of the markedly increased ETP values and hypocoagulable because of the impairment of fibrinogen cleavage (in vitro models of homozygotes).

The resistance to thrombin inactivation by antithrombin is the main effect of this novel F2 mutation, which is in agreement with some of the findings of the TG. In the absence of heparin, RRTA measured in plasma of probands and their affected relatives after addition of antithrombin were only slightly higher than in members without the mutation. In contrast, in the presence of heparin, all carriers of the mutation showed a clear antithrombin resistance, particularly during the first 5 minutes of the inactivation reaction.
screening strategy, based only on the personal history of provoked or unprovoked VTE and the presence of an isolated mild reduction of the FII:Act with normal PT followed by gene sequencing, we identified 2 carriers of prothrombin Padua 2 out of 400 VTE patients with an approximated prevalence of 5 carriers in 1000 patients (0.5%). Whether this is an over or under-estimation because of some selection or referral bias needs to be confirmed by studies in different cohorts and in different populations, and our estimate must be considered preliminary.

Interestingly enough, in a genome-wide linkage scan in thrombophilic families, performed to identify novel susceptibility regions for VTE (GENUT study), ten Kate et al reported a missense mutation in F2 characterized by the replacement of an Arginine by Tryptophan (the authors did not indicate the amino acid position). Whether this mutation corresponds to prothrombin Padua 2 remains to be defined.

In conclusion, substitutions at Arg596 of the prothrombin molecule, such as prothrombin Padua 2, constitute a new and relatively frequent cause of inherited thrombophilia.

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Disclosures
None.

Significance
We studied a novel substitution affecting Arg596 of prothrombin (p.Arg596Trp or prothrombin Padua 2) in 2 Italian families. This new prothrombin variant resulted in antithrombin resistance and increased risk of venous thromboembolism in carriers. In a series of 400 unrelated consecutive patients with venous thromboembolism, we found 2 carriers of prothrombin Padua 2 (estimated prevalence of 0.5%). Interestingly, some authors reported in a genome-wide linkage scan in thrombophilic families, performed to identify novel susceptibility regions for venous thromboembolism (GENUT study), a missense mutation in F2 characterized by the replacement of an Arginine by Tryptophan (the amino acid position was not indicated). Whether this mutation corresponds to prothrombin Padua 2 remains to be defined. Substitutions at Arg596 of the prothrombin molecule, such as prothrombin Padua 2, constitute a new and relatively frequent cause of inherited thrombophilia.
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Materials and Methods

Coagulation tests
After obtained informed consent, venous blood was drawn from probands and their relatives in 3.8% sodium citrate (9/1, v/v). Platelet-poor plasma (PPP) was stored in aliquots at -80°C until tested. Genomic DNA was extracted from whole blood using an automated method (MagCore Extractor System H16, RBC Bioscience, New Taipei City, Taiwan).
PT, activated partial thromboplastin time (aPTT), fibrinogen concentration, plasminogen activity, AT activity, protein C (PC) antigen, PC coagulometric and chromogenic activities, total and free protein S (PS) antigen, PS coagulometric activity, lupus anticoagulant (LA), anti-cardiolipin and anti-beta2-glycoprotein I antibodies and activated protein C (APC) resistance were determined as previously described.1-5 Factor VIII, IX and XI activities were measured by an aPTT-based clotting assay (Dade® Actin® Activated Cephaloplastin reagent, Siemens, Muenchen, Germany), while factor X activity was assayed using a PT-based clotting assay (Thromborel® S, Siemens) on a BCS® XP coagulometer (Siemens) with the relative factor-deficient plasma (Siemens). Calibration curves were prepared by a lyophilized normal pooled plasma (Standard Human Plasma, Siemens). Normal ranges were evaluated in 100 healthy subjects of both sexes, aged 20–70 years. Pregnant women and women under oral contraceptives were excluded as well as subjects taking any medication. Values were normally distributed and normal ranges were calculated as mean ± 2 standard deviation (SD). Factor V Leiden and prothrombin G20210A polymorphisms were detected as previously described.3,6

Assays for prothrombin activity and antigen
Prothrombin activity (FII:Act) was measured with a PT-based clotting assay using Thromborel® S (Siemens) and prothrombin-deficient plasma (Siemens) on a BCS® XP coagulometer (Siemens). FII:Act was also evaluated by a modification of the ecarin clotting time (ECT). The measurement was performed on a Mechrolab Clot-Timer (Heller Laboratories, Santa Rosa, CA, USA) according to Girolami et al.7 by the addition of 50 µl of a 1:10 dilution of the test plasma, 50 µl of a mixture containing equal volumes of ecarin (Diagnostica Stago, Asnières-sur-Seine, France) and cephaloplastin (Dade® Actin® Activated Cephaloplastin reagent, Siemens) and 50 µl of 25 mM CaCl2 to 50 µl of prothrombin-deficient plasma (Siemens). The final concentration of ecarin was 2.5 U/ml. Prothrombin antigen (FII:Ag) was determined by an in house sandwich enzyme-linked immunosorbent assay (ELISA) as previously reported.6 Calibration curves and normal ranges were generated as mentioned above.

F2 gene analysis
All 14 exons, splice junctions and the 5’ and 3’ untranslated regions (UTRs) of F2 (NCBI Reference Sequence: NG_008953.1) were amplified from genomic DNA by polymerase chain reaction (PCR) and sequenced directly with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM® 3100 automated sequencer (Applied Biosystems). To exclude that the new F2 variant was a polymorphism, 100 healthy subjects (50 men and 50 women, between 20 and 80 years of age) and 100 patients (50 men and 50 women, between 20 and 80 years of age) with documented DVT, followed in our Thrombosis Centre, were investigated by sequencing of the exon in which the variation
was found. A different group of 400 consecutive patients with provoked and unprovoked VTE (in a ratio 2:3), referred to our Centre for coagulation screening, were tested for FII:Act levels (PT-based assay) and PT. In patients with levels of FII:Act below the lower limit of the normal range (FII:Act <80% in those not under oral anticoagulation) and with a normal PT, gene analysis was performed to identify prothrombin variants at Arg596.

Restriction enzyme analysis
The F2 variant identified by sequencing was confirmed by digestion of PCR-amplified DNA with the restriction enzyme HpaII (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. The digestion products were separated by 4% (w/v) agarose gel electrophoresis.

Recombinant prothrombin Padua 2 expression
The expression vector pCDNA™3.1(+), containing the full-length cDNA of human prothrombin (Invitrogen, Carlsbad, CA, USA), was used as template for the introduction of the mutation by the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). Introduced substitution was confirmed by sequencing. Human Embryonic Kidney 293 (HEK293) cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum during transfection and selection. Cells were transfected with wild type and mutant expression vectors by TransIT®-LT1 Transfection Reagent (Mirus, WI, USA) according to the manufacturer's instructions. Stable cell clones, selected using hygromycin B (Invitrogen), were assayed for their ability to produce prothrombin by ELISA. Clones expressing high levels of wild type and mutant recombinant prothrombins (WT rFII and Arg596Trp rFII, respectively) were expanded in serum-free medium supplemented with insulin-transferrin-selenium (Gibco®, Grand Island, NY, USA) and 10 μg/ml vitamin K1 for 24 h. Conditioned media containing recombinant prothrombins were harvested and centrifuged at 2,000 x g for 10 min to remove cellular debris. The supernatants were treated with 5 mM benzamidine and stored at -80°C until used. One thawed, the supernatants were concentrated using Amicon Ultra-15 centrifugal filter devices (MWCO of 30 kDa, Merck Millipore, Darmstadt, Germany), dialyzed against TBS (20 mM Tris-HCl and 150 mM NaCl, pH 7.4) containing 5 mM benzamidine and applied at 4°C to a Q-Sepharose Fast Flow column (GE Healthcare, Milano, Italy), previously equilibrated with TBS supplemented with 5 mM benzamidine. The column was washed with the same buffer and bound proteins were eluted with 20 mM Tris-HCl and 1 M NaCl, pH 7.4. Fractions with positive Bradford assay were pooled, dialyzed again and stored at -80°C. Both preparations, partially purified, were analyzed by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (Bio-rad, Hercules, CA, USA). Band of each recombinant prothrombin was compared to that of normal prothrombin purified from human plasma (Haematologic Technologies, Essex Junction, VT, USA).

Antigen and activity levels for recombinant prothrombins were evaluated as described above. FII:Act was measured with both methods after reconstitution of prothrombin-deficient plasma with WT rFII or Arg596Trp rFII at a final concentration of 90 μg/ml, corresponding to the prothrombin concentration in normal human plasma (100% of normal). Calibration curves were obtained with a normal pooled plasma (Siemens).

Thrombin generation assay

*Patients' plasma*
Thrombin generation (TG) was determined in PPP with the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands) as previously described.8,9 TG was triggered with both 5 pM and 1 pM TF concentrations. Four TG
parameters were analyzed: lag time, endogenous thrombin potential (ETP), peak height and decay (expressed as “start tail”: time from the start of the reaction to when the TG curve touches zero at the end of the reaction).

Recombinant prothrombins
TG was measured in prothrombin-deficient plasma reconstituted with WT rFII or Arg596Trp rFII at a final concentration of 90 µg/ml, (100% of normal). The heterozygous condition was obtained by reconstituting prothrombin-deficient plasma with 50% WT rFII and 50% Arg596Trp rFII.

Antithrombin resistance assay
Patients’ plasma
The ability of AT to inactivate thrombin in plasma samples was evaluated. Plasma was diluted 1:100 with a buffer containing 50 mM Tris-HCl and 200 mM NaCl, pH 8.1 (reaction buffer), activated to thrombin by ecarin and then inactivated by AT. The activation assay was carried out as follows: 250 µl of a 1:100 dilution of plasma was mixed with 25 µl of an aPTT reagent (Dade® Actin®, Activated Cephaloplastin, Siemens), 25 µl of 25 mM CaCl₂ and 50 µl of 1.1 U/ml ecarin (Diagnostica Stago). Samples were incubated at 37°C for 2 min. Once formed, thrombin was inhibited by 50 µl of 0.5 U/ml AT (Kedrion, Lucca, Italy) in the absence and presence of 1 U/ml heparin. Residual thrombin activity was evaluated during time by the addition of 100 µl of 0.5 mM chromogenic thrombin substrate S-2238 (Sekisui Diagnostics, Stamford, CT, USA). After 2 min, substrate hydrolysis was quenched with 50 µl of 10% acetic acid. An aliquot of each reaction mixture was transferred to a microtiter plate and the absorbance was read at 405 nm. Relative residual thrombin activity (RRTA) was calculated by comparison with data obtained at 0 min.

Recombinant prothrombins
AT resistance of recombinant prothrombins was also analyzed. Each recombinant prothrombin was diluted to 0.9 µg/ml with the reaction buffer before being added to the mixture containing phospholipids, CaCl₂, ecarin and AT. For recombinant prothrombins, AT resistance assay was performed with 50 mU/ml ecarin and 100 mU/ml AT in the absence and presence of 5 U/ml heparin. RRTA was calculated as mentioned above.

Detection of thrombin-antithrombin complexes
Patients’ plasma
Thrombin-antithrombin (TAT) complexes levels were measured in plasma by a commercially available ELISA kit (Enzygnost® TAT micro, Siemens) following the manufacturer’s recommendations. Reference standards were included in the kit. Reference values for the normal population were obtained as described above.

Recombinant prothrombins
The binding between recombinant prothrombins and AT was quantified by the TAT complexes assay. Recombinant prothrombins were converted into thrombins by ecarin and then incubated with AT in the absence and presence of heparin as described before. Reactions were quenched over time by the addition of 100 µM of D-phenylalanyl-L-prolyl-L-arginine-chloromethylketone (PPACK, Haematologic Technologies). Reference standards were those included in the ELISA kit.

Cleavage of fibrinogen by recombinant (pro)thrombins with a clotting method
The ability of recombinant thrombins to cleave fibrinogen to fibrin was determined with a clotting method in an isolated model in vitro. Briefly, 0.7 µg of each recombinant prothrombin in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4, was incubated for 30 min at 37°C with 7.5 µl of phospholipids (Dade® Actin® Activated Cephaloplastin reagent,
Siemens), 15 µl of 25 mM CaCl$_2$ and 50 µl of 2.5 U/ml ecarin (Diagnostica Stago) in a total volume of 150 µl. After 30 min incubation at 37°C, 100 µl of 2.5 mg/ml fibrinogen (Haematologic Technologies) was added to the activation mixtures and the clotting time (in sec) was measured on a Mechrolab Clot-Timer (Heller Laboratories).
References


