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Conformational Asn187Asp/Lys antithrombin variants and thrombosis

Clinical and biological features in 13 new heterozygotes

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Summary

Antithrombin Rouen VI (N187D) is a rare conformational thermolabile variant. The unique symptomatic carrier reported in the literature developed 3 thrombotic events during pregnancy, in each case in a context of pyrexial infection. In fresh plasma, antithrombin activity and antigen level were normal but *in vitro* experiments demonstrated the presence of a thermolabile variant, suggesting that fever could be a trigger for thrombosis in N187D carriers. The Rouen VI variant was further found in two asymptomatic brothers. In these subjects, it was associated with normal antigen level but reduced activity. In order to better delineate the functional and clinical consequences of the N187 variants, we have studied a series of seven subjects from two distinct families heterozygous for the Rouen VI mutation. Antithrombin levels were normal or borderline in these patients. Thermostability of plasma antithrombin was normal. We have also studied six subjects heterozygous for a new mutation, 6462C>G, which results in an asparagine to lysine substitution at residue 187. In these patients, the N187K mutation is associated with a clear type II deficiency and decreased thermostability of the plasma protein has been demonstrated. That the N187D mutation has milder consequences on plasma antithrombin activity than the N187K mutation is in agreement with structural predictions. About 50% of the N187 carriers studied have suffered venous thrombotic events, strongly suggesting that both mutations are risk factors for thrombosis, but none occurred during pyrexial infections.

Keywords

Antithrombin, conformational variant, thrombophilia, serpin

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Introduction

Antithrombin (AT) is a major physiological inhibitor of the coagulation process. This 58-kD plasma protein inhibits the procoagulant serine proteases thrombin and factor Xa. Formation of inhibitor-protease complexes is highly accelerated by heparin or by heparan sulfate glycosaminoglycans lining the vascular endothelium. Inherited AT deficiency is associated with a 25– to 50-fold increase in the risk of venous thromboembolism (VTE) (1–4). It is found in between 2 and 5% of young adults presenting with a history of venous thrombosis. AT deficiency is inherited as an autosomal dominant trait and most deficient subjects are heterozygotes. More than 150 'private' mutations have been described in type I (quantitative) or type II (qualitative) AT deficiencies (5). Type I AT deficiency is characterized by a similar decrease in AT activity and antigen levels. Type II AT deficiency is defined by the presence of circulating abnormal AT molecules. In type II AT deficiency, the risk of thrombosis depends on which function of the protein is modified. For instance, heterozygosity for 'active site' AT variants strongly increase the risk of thrombosis, but heterozygosity for 'heparin binding site' variants does not (5).

'Conformational' AT mutations have only been identified more recently, probably because they do not always induce clear modifications of plasma AT levels. Reports of families carrying such variants are rare and the risk of thrombosis associated with their presence is not clearly quantified (6, 7). The first conformational variant described was AT Rouen VI (N187D). This variant was identified in a woman who experienced three episodes of deep vein thrombosis (DVT) preceded by pyrexial infections

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during pregnancy. This woman had normal plasma AT activity and antigen levels. Structural and functional investigations performed on the purified N187D AT variant demonstrated that the mutation modifies the three-dimensional structure of the protein, which has an accelerated thermal lability: the inhibitory activity of the purified variant decreases at 40°C (fever temperature) and this modification is associated with the formation of latent and polymerized AT molecules (7). Because each episode of thrombosis occurred during pyrexial infections, it was suggested that fever could be the main trigger of the clotting process. The N187D mutation was further found in two brothers from an unrelated family (6). Heterozygosity for the mutation was associated here with a clear type II deficiency. However, both subjects were asymptomatic. Another variant at residue 187 (N187K resulting from the 6462C>A mutation) has been also reported. The N187K mutation was found in an asymptomatic adult presenting with a mild AT deficiency. Characterization of the variant has not been performed.

The aim of our study was to gain more insight into the clinical and biological consequences of AT mutations at residue 187. We have recorded clinical and biological data for thirteen new heterozygotes from three unrelated families who carry N187D or N187K AT mutations. We have also investigated AT thermostability as well as the presence of latent or polymerized AT in plasma.

Patients and methods

DNA studies

Probands from family 1 to 3 were investigated following spontaneous thrombotic episodes and available family members were then studied. All subjects gave their informed consent to genetic analysis. DNA was isolated using Miller's method (11) or with a blood DNA purification kit (GFX genomic, Amersham Biosciences) and stored at 4°C. The seven AT exons and intron-exon junctions were amplified by PCR as previously described (12), primer sequences and amplification program are available on request. PCR products (8 µl) were submitted to digestion with exonuclease I (10 U) and shrimp alkaline phosphatase (2U) for 15 min at 37°C, using a PCR product pre-sequencing kit (USB, Amersham Biosciences). After enzyme inactivation, cycle sequencing was performed in 96-well plates using the same set of primers and the Big dye Terminator Cycle v.2 sequencing kit (PE Applied Biosystems, Warrington, UK) for 25 cycles (95°C 30 s, 58°C 15 s, 60°C 4 min). After purification by gel exclusion (Sephadex G50, Amersham Biosciences) on 96-well plates (Multiscreen, Millipore), the reaction products were analyzed on an ABI prism 3700 DNA analyzer (Applied Biosystems).

Plasma AT assays

Citrated venous blood was drawn and plasma samples were immediately aliquoted and stored at –80°C. For consistency, all the assays were performed on frozen plasma. Under these conditions, we verified that AT activity was stable during at least one year. Heparin cofactor activity was measured with an amidolytic assay using the synthetic chromogenic substrate CBS 61.50 and bovine thrombin (Stachrom AT) on an STA analyzer (both from Diagnostica Stago, Asnières, France). Immunoreactive AT was measured by Laurell immunoelectrophoresis using sheep polyclonal anti-human-AT IgG antibodies (The Binding Site, Birmingham, UK). Crossed immunoelectrophoresis was performed with 10 U/ml heparin in the first dimension, as described by Sas et al. (13).

Non denaturing PAGE was performed using 8% gel as previously described (14). Urea-PAGE was performed using the non denaturing PAGE procedure excepted that 6M electrophoresis grade urea (Sigma) was added to the gel. Purified human native and latent AT were used as controls. Human AT was purified from citrated frozen plasma, essentially according to McKay (15). Latent AT was prepared as previously described (16). Proteins were transferred to nitrocellulose membranes (Hybond-C extra, Amersham Biosciences). The membranes were saturated with 5% non fat dried milk and incubated with polyclonal anti-AT sheep IgGs diluted 1/5000 in Tris-buffered saline/Tween 0.5% for 2 h at room temperature, then washed and incubated with a peroxidase-labeled donkey anti-sheep IgG antibody diluted 1/10 000 in Tris-buffered saline/Tween 0.5% (both antibodies from The Binding Site, Birmingham, UK). AT was revealed by chemoluminescence (ECL, Amersham Biosciences).

The heat stability of N187D and N187K ATs was tested by incubating undiluted plasma samples from four heterozygous subjects and four normal subjects for 24, 72 or 144 h at 40°C. AT heparin cofactor activity was then measured, as described above.

Results

Case reports

Data are summarized in Table 1. Protein C and protein S activity and antigen level, FV Leiden, prothrombin G20210A mutation, and the presence of antiphospholipid antibodies were investigated in each symptomatic member of these families. None of these genetic or acquired thrombosis risk factors was present.

Family 1

The proband was a 33-year-old Caucasian woman from Belgium, who suffered from a spontaneous massive iliofemoral DVT. There was no history of thrombosis in her parents or in the proband's five brothers and sisters. One year later, during the fourth month of her third pregnancy, she suffered from a pulmonary embolism (PE) . AT deficiency was diagnosed (73 and 85% for AT activity and antigen respectively, normal range 80–120%). Analysis of the antithrombin gene indicated that she was heterozygous for the 6460A>G substitution, leading to the Rouen VI (N187D) AT variant. Family study showed that the mutation had been transmitted by her mother, who had had six children and was asymptomatic. One sister of the proband, who was 45 and had two children, and one 15-year-old son of the proband, both asymptomatic, were heterozygous for the mutation. The proband is now 44 years old and receiving life-long oral anticoagulation. Four non-affected asymptomatic family members were also investigated as shown in figure 1.

Family 2

The proband was a 33-year-old Caucasian woman from the north of France, who suffered from iliac DVT during the post-partum period of her third pregnancy. Two earlier pregnancies had been

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Table 1: Family history of thrombosis in thirteen new and four already described heterozygous carriers of N187D/K AT variants.

AT Act/Ag	Subject	Sex	Age	Thrombosis	Recurrence	Situations at risk for VT
N187D AT (6460A>G)						
• **Family 1**						
73/85	proband	f	33 34	iliofemoral DVT PE	yes	0 3rd pregnancy
80/89	mother	f	-	0		6 pregnancies
75/84	sister	f	-	0		2 pregnancies
78/82	son	m	-	0		0
• **Family 2**						
70/79						
79/97	proband	f	33	iliac DVT	no	post partum (3rd pregnancy)
74/88	sister	f	27 31	SVT SVT	yes	2nd pregnancy post partum (3rd pregnancy)
76/88	sister	f	23	DVT	no	1st pregnancy (2 pregnancies)
Already described						
• ***Rouen VI proband (6,7)***						
85/96	*proband*	*f*	*29* *?* *?*	*DVT* *DVT + fetal loss* *DVT + fetal loss*	*yes* *yes*	*first pregnancy + fever* *2nd pregnancy + fever* *3rd pregnancy + fever*
• ***Two brothers (6)***						
51/100	*one subject*	*m*	*-*	*0*		
58/112	*brother*	*m*	*-*	*0*		
N187K AT						
• **Family 3 (6462C>G)**						
55/78	proband	f	18	DVT proximal + PE	no	oral contraception (since 18 months)
59/71	mother	f	-	0		1 pregnancy
62/76	uncle	m	44 49	iliofemoral DVT VT + PE	yes	orthopedic surgery 5h long trip by plane
53/65	uncle	m	42 44 50	distal VT distal VT distal VT	yes yes	vascular surgery 0 0
nd/nd	uncle	m		0		
nd/nd	cousin	f		0		
• ***Already described (6462C>A) (6)***						
73/70	*one subject*	*m*		*0*		

AT Act : AT heparin cofactor activity (normal range 80-120%), AT Ag : AT antigen (normal range 80-120%), S/DVT: superficial/deep venous thrombosis, PE pulmonary embolism

carried out without any problem and she had received an oral contraceptive (OC) treatment for five years without complication. A mild AT deficiency was found (heparin cofactor activity level 70%, antigen level 79%). Analysis of the AT gene indicated that she and her two sisters were heterozygous for the Rouen VI mutation. The younger sister experienced one episode of DVT at age 23, during the fourth month of her first pregnancy. She had a normal second pregnancy without any prophylactic treatment. The older sister developed a superficial venous thrombosis (SVT) during the third trimester of her second pregnancy, as she was 27 years old, and a second SVT during the postpartum period of her third pregnancy four years later. The parents were asymptomatic.

Family 3

The proband was a 18-year-old Caucasian woman from the north of France, who experienced DVT and PE 18 months after initiation of an OC treatment. Her AT heparin cofactor activity was 55% and AT antigen level 78%. She turned out to be heterozygous for a new 6462C>G AT gene mutation, leading to a N187K AT variant. Her parents were asymptomatic. Her mother, three uncles and a cousin were also heterozygous for the mutation. One uncle developed a DVT at age 44 after orthopedic surgery and a PE 5 years later after a 5-hour-long trip by plane. The sec-

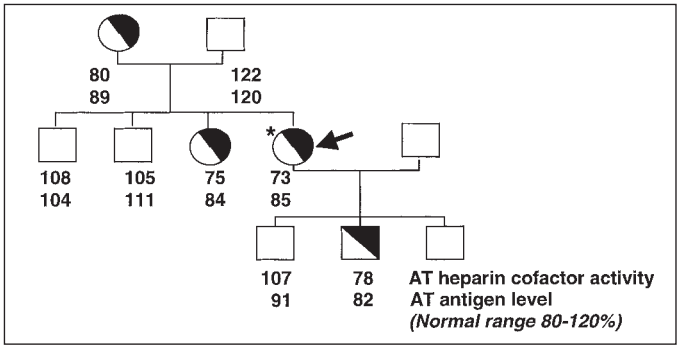

Figure 1: Pedigree of family 1. AT heparin cofactor activity and antigen levels of N187D AT heterozygotes or non-affected members are indicated. The proband is indicated by an arrow, the asterisk indicates a symptomatic subject.

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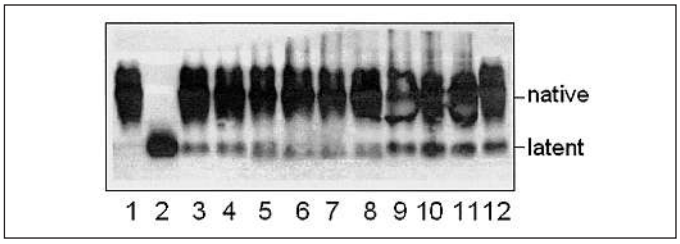

Figure 2: Urea-PAGE analysis of latent AT in N187D/K AT heterozygote's plasma, showing the migration of purified native AT (lane 1), purified latent AT (lane 2), normal plasmas (lane 3–4), N187D (lane 5–8) and 187K (lane 9–12) heterozygote's plasmas.

ond uncle suffered from a distal VT after vascular surgery at age 42, and from two spontaneous VT episodes which occurred at ages 44 and 50. The youngest uncle, who was 43, and his eleven-year-old daughter were asymptomatic. It is likely that the mutation was transmitted by the grand father on the maternal side who had a low plasma AT heparin cofactor activity (57%) but his DNA was not available for gene analysis.

Plasma phenotype

AT activity and antigen level

In seven N187D heterozygotes from two unrelated families, AT antigen level consistently showed values in the lower normal range, (median 86.5%, range 79–97%, normal range 80–120%) (Table 1). Heparin cofactor activity was slightly decreased, about 10% lower than the antigen level (median 75.5%, range 70–80%, normal range 80–120%). Overall, plasma phenotypes indicated a very mild AT deficiency excepted in one subject with AT levels in the normal range. Interestingly, in family 1, four wild type subjects were also investigated (Fig. 1). Non-affected members had a median AT activity level about 30% higher than heterozygotes (107.5%, range 105–122%, versus 76.5%, range 73–80% respectively) and a median antigen level about 20% higher than heterozygotes (107.5%, range 91–120% versus 84.5%, range 82–89%). This indicated that the Rouen VI mutation affects both AT antigen and AT activity.

The four N187K heterozygotes had a clear qualitative AT deficiency (Table 1). Median AT heparin cofactor activity was 57%, (range 53–62%) and median antigen levels was 72% (range 65–78%).

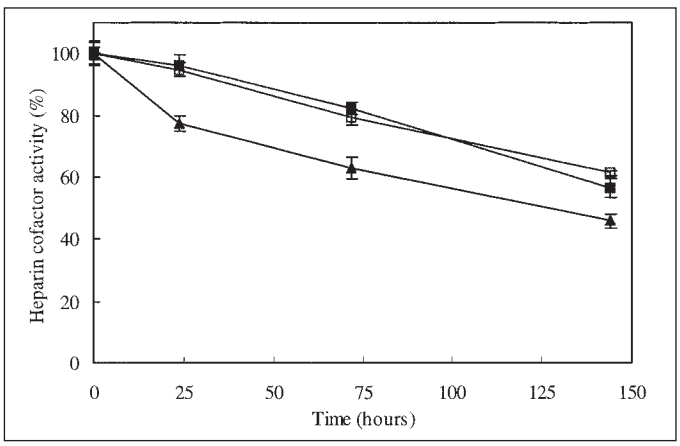

Figure 3: Thermostability of plasma AT from N187D (open square) or N187K (black triangle) heterozygotes compared with normal plasma AT (black square). For heterozygotes or controls, plasmas from four individuals were heated at 40°C for 24, 72 or 144h and the remaining heparin cofactor activity was expressed as a percentage of the initial activity (mean ± SD).

Electrophoretic analyses

Crossed-immunoelectrophoresis (CIE) performed on plasma of N187D or N187K heterozygotes in the presence of heparin consistently displayed an abnormal profile. In most cases, two peaks were observed: a major normal peak and a minor peak with decreased heparin affinity. An unusual profile was obtained with the N187K proband's plasma since, in addition to the major normal peak, two peaks with low heparin affinity were present. Only one low-heparin affinity peak was observed in samples from other N187K heterozygotes.

PAGE (polyacrylamide gel electrophoresis) analysis was performed on plasma from four N187D and four N187K heterozygotes. The migration pattern could not be distinguished from that of control plasma or purified normal AT with the exception of a slightly more anodal migration for the plasma containing the 187D AT variant (not shown). Polymerized AT could not be detected in plasmas from heterozygotes.

Since latent and native AT might not be separated by PAGE, the same plasmas were tested on urea-PAGE in order to clearly identify latent AT (Fig. 2). Results of this semi-quantitative analysis showed that latent AT was present in normal plasma, as previously described (8). Latent AT was also present in N187D samples, where it migrated as a double band consistent with the more negatively charged N187D having a more anodal migration than wild type AT. Latent AT levels were higher in N187K plasmas, but not in N187D plasmas. The latent N187K variant migration profile was not different from that of the wild type latent AT.

Thermostability

It has previously been reported that the N187D variant is a thermolabile protein able to loose activity at fever temperature, and we hypothesized that the N187K AT is also a thermolabile variant. Results of the thermostability study, performed on plasmas from heterozygotes for the N187D or N187K mutants, are shown in figure 3. In N187D plasma and in control plasma, modifications in heparin cofactor activity induced by heating were very similar. However, in N187K plasmas, a significant decrease in thermal stability was observed, with a 23% mean decrease in heparin cofactor activity after 24h at 40°C for the heterozygotes, versus 6% for the normal controls. The difference in thermostability was confirmed after 72 or 144h heating.

Discussion

Ten years ago, AT Rouen VI (N187D) was described as a conformational or 'unstable' AT variant. This variant was first described in a patient who had suffered from thrombotic events associated with pyrexial infections. It was suggested that thrombotic events in this patient could be induced by loss of activity of

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the variant at increased body temperature (7). However, the same mutation was also found in two asymptomatic blood donor brothers with no family history of thrombosis (6). Since then, very few conformational AT variants have been described, although a number of already known ATs, such as the pleiotropic variants, are suspected to share this structural instability (17). Reasons for that might be: i/ AT unstable variants are very rare ii/ they remain unrecognized because they cause a very mild deficiency or even no plasma AT deficiency at all iii/ they are associated with a weak thrombotic risk. In fact, if the structural and biochemical properties of several unstable ATs were investigated in details, the *in vivo* consequences of such deficiencies deserve further investigations.

We analysed the clinical and biological features of thirteen heterozygotes from three unrelated families who carry an unstable AT caused by mutations at position 187. The Rouen VI mutation (6460A>G, N187D) was found in seven subjects, six women and one young man, from two unrelated families, so that, altogether, ten heterozygous carriers of the Rouen VI mutation from four families are now reported (Table 1). It is noteworthy that family 1, 2 and the Rouen VI first proband originate from the same geographic area (north of France or Belgium), and that two other affected brothers are from Scotland, which suggests a possible founder effect. A new point mutation, 6462C>G that leads to a N187K AT variant, was identified in family 3. Interestingly, the same AT variant was previously described in one individual who carries another mutation (C>A) in the same codon (Table 1) (6).

Our data are consistent with initial findings concerning the Rouen VI proband and confirm that the N187D variant is associated with a very mild or no AT deficiency contrasting with a high prevalence of thrombotic events in the selected families (6, 7). It is difficult to estimate the increase in the risk for venous thrombosis in N187D AT carriers since i/ only four families were described to carry this mutation, ii/ most families (3/4) were selected through a symptomatic proband and iii/ the prevalence of this mutation in the general population is not known. However, the rare occurrence of the mutation strongly suggests that it is indeed a thrombosis risk factor.

The N187D substitution does affect the antigen level as demonstrated in family 1 where AT antigen level was about 20% lower in heterozygotes than in normal family members. This might be explained by an increased turn-over and/or a decreased secretion of the unstable AT variant. In two N187D heterozygotes previously reported by others, normal antigen level (100 and 112%) but clearly decreased AT heparin cofactor activity (51 and 58 %) were found (6). Difference in the assays used or rapid degradation of AT during handling or storage due to its low stability might be responsible for this discrepancy. Plasmas samples studied here were prepared and frozen immediately after blood sampling. Under these conditions, we verified that heparin cofactor activity remains unchanged over time.

In N187D heterozygotes, 50% (5/10) of the subjects studied suffered from VTE (one PE, six DVT and two SVT). In most cases the first thrombotic event occurred in the late twenties or thirties. Thrombotic events in this series were not as severe as those described in the Rouen VI proband, who experienced three DVT with fetal loss during pregnancy-associated pyrexial infection (6, 7). Because of the thermolabile property of the purified variant, fever was proposed to be a specific thrombosis risk factor for N187D carriers (7, 17). This could not be confirmed in this series since no pyrexial episode was recorded before or during the thrombotic episodes. However, a feature common to these cases and the Rouen VI first proband was that five out of six episodes were related to pregnancy or post-partum. Only one spontaneous DVT was recorded.

Pregnancy is a well known risk factor for VTE. Incidence of pregnancy-associated VTE in the general population is estimated to be 0.5–1 per thousand with a five to six times higher risk in pregnant versus non pregnant women of the same age (18–22). The risk of VTE in type I AT-deficient pregnant women not receiving anticoagulant therapy is high: in a study by Conard et al., 62 to 68% of women carrying a type I AT deficiency suffered from DVT during pregnancy (20). In the present series, 6 women heterozygous for N187D had 19 pregnancies and only 5 pregnancies were associated with SVT or DVT, which suggests a lower risk of thrombosis associated with this mutation than with type I AT deficiency. Nonetheless, pregnancy is a situation at risk for thrombosis in N187D carriers. Increased levels of prothrombin F1+2, fibrinopeptide A, thrombin-antithrombin complexes and D-dimers indicate an activation of blood coagulation during normal pregnancy (23, 24). One hypothesis is that increased AT turn-over in heterozygous pregnant women increases the deficiency state and the risk of thrombosis.

Unlike the Rouen VI AT mutation, the N187K variant induces a type II AT deficiency with decreased plasma AT activity, and a clear discrepancy between activity and antigen level (Table 1). So far, only one heterozygote carrying a 6462C>A mutation leading to the presence of a N187K AT variant has been previously reported. This subject was asymptomatic. Heterozygotes in family 3 carry another mutation (6462C>G) although leading to the same variant. In this family, six thrombotic events occurred in three heterozygotes and three subjects were asymptomatic (Table 1). In four cases, thrombosis occurred in the presence of an environmental risk factor, OC treatment, orthopedic or vascular surgery or long trip by plane, which are well-known situations which increase the risk of VTE. Fever was not found to be associated with thrombosis occurrence.

Asparagine 187 is located on helix F and is a highly conserved residue among serpins (25). Structural and experimental data indicate an important role for helix F in serpin stability and inhibitory activity (26). Asparagine 187 forms a key hydrogen bond with a carbonyl group of Ile 202 in the loop between helix F and s3A which is connected to the A sheet through s5A (26, 27). These interactions stabilize helix F and the overall serpin structure. Substitution of asparagine 187 by a glutamate eliminates this hydrogen bond causing a destabilization of the molecule. In the N187K variant, disruption of the N187-Ile202 hydrogen bond in addition to a large positively charged side chain is likely to result in stronger alteration in protein structure or folding. This is consistent with the deeper AT deficiency observed in 187K carriers. Purified N187D AT has a slightly more anodal migration than normal AT, that we could observe on urea-PAGE (Fig. 2). It has been previously demonstrated that this variant has an increased heparin affinity and normal inhibitory activity which deteriorates slowly on storage at 4°C and more rapidly on incubation at 37°C or 41°C (7). This is associated, *in vitro*,

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with formation of latent AT and of short chain polymers. We investigated if these forms were present in plasmas from 187D and 187K carriers. AT polymers were not detected, however, latent AT was clearly present in all the plasmas, including control plasma, but was found at a higher level in 187K carriers only. In addition, we were able to observe a decreased AT thermostability at 40°C in N187K but not in N187D plasma. Altogether, these results are consistent with structural predictions, the K substitution being more detrimental than the D substitution.

One puzzling issue is that we did not observe any increase in AT thermolability in N187D plasma although the purified AT N187D variant was described to be thermolabile at 37 or 40°C (7). Several reasons may account for this. First, the assays were performed in quite different conditions: it is likely that the protein stability is different in purified solution at 1g/l and in plasma at a 10-fold lower concentration. In addition, both normal and variant AT are present in heterozygote's plasma. Normal AT accounts for at least 50% of the protein, but the exact proportion of variant AT is unknown. In these conditions, our assay might not be sensitive enough to detect a modest decrease in thermostability. It can be hypothesized that a higher proportion of variant in the Rouen VI proband's plasma results in a higher plasma AT lability. Interestingly, we previously made a similar observation with another unstable AT variant, AT F229L. Thermostability of AT in plasma from F229L heterozygotes is normal. By contrast, in plasma from a homozygote, thermostability of AT is clearly decreased (9). Finally our assays, for consistency and practical reasons, were performed on frozen plasmas and this might be another explanation.

Overall, the N187D AT variant certainly is a risk factor for venous thrombosis even if it is associated with borderline or even normal AT plasma levels. A N187K AT variant resulting from a new mutation was also identified in subjects with VTE. For both variants, thrombotic events occurred most often in clinical situations at risk for thrombosis, such as pregnancy or surgery. This is consistent with previous reports of other conformational AT variants: AT Wibble (T85M), AT Wobble (T85K) and AT F229L (6–8). In carriers of these variants, no spontaneous thrombotic event is described. In the Wibble family, thrombosis occurred only in family members who had two additional genetic risk factors, FV Leiden and a protein S C145Y variant. For AT Wobble and AT F229L, only the probands (a 10-year-old girl with a severe chest infection, and a homozygous one year-old infant who suffered from a venous cerebral thrombosis respectively) had a thrombotic history (8, 9). Finally, we could not confirm the role of fever as a specific risk factor. Although it may contribute to thrombosis occurrence or severity as previously suggested, it does not represent here a condition frequently associated with thrombosis.

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