

### Identification of six novel mutations in type I antithrombin deficient Italian families

In 8 patients with antithrombin (AT) deficiency with deep vein thrombosis, belonging to unrelated kindreds, we identified 8 different point mutations. Only two out of the 8 mutations identified have been previously reported. The spectrum of unreported AT mutations identified may help in unravelling the role of specific regions of the AT gene.

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Antithrombin (AT) is the most important inhibitor of thrombin and factor X and one of the major physiologic inhibitors of hemostasis.<sup>1</sup> Hereditary AT deficiency is an autosomal dominant disease occurring in up to 6% of thromboembolic patients.<sup>2</sup> Type I AT is defined by concordantly low circulating levels of both functional and immunological AT, whereas type II AT deficiency corresponds to the presence of an abnormal protein in the bloodstream. Identification of gene mutations occurring in AT-deficient patients has given important information on the structure-function relationships of this hemostatic protein.

We investigated 8 Caucasian subjects, belonging to 8 unrelated families, with a confirmed diagnosis of AT deficiency looking for gene alterations within the AT gene. We also investigated the subjects' first-degree relatives, when

available. Blood samples were collected and tested for antiphospholipid antibodies, antithrombin activity and immunoreactivity (ELISA, Diagnostica Stago, Asnières, France), protein C (amidolytic and immunological assays; Behring, Marburg, Germany) and total and free protein S antigen (ELISA, Diagnostica Stago, Asnières, France), as reported elsewhere.<sup>3</sup> Isolation of DNA and FV Leiden and FII A20210 mutation analysis were done as previously described.<sup>3</sup> All coding regions of the AT gene and intron/exon boundaries were amplified using sense and antisense oligonucleotides designed on the basis of known sequences of the AT gene locus (Genbank accession number X68793). Amplified DNA fragments were purified and subjected to direct cycle sequence analysis using the Taq dye-deoxy terminator method and an ABI PRISM 3100 Genetic Analyzer sequencer (PE Biosystems, USA). The 8 different heterozygous mutations we identified within the AT gene are reported in Table 2. We found 2 missense mutations, 2 nonsense mutations, 1 insertion of two nucleotides, 1 deletion of a single nucleotide, and 2 mutations that alter the pattern of mRNA processing. None of the mutations identified was found in unaffected relatives. To exclude the possibility that one or more of mutations identified could be a polymorphism, all mutations were further investigated in another 100, apparently healthy, subjects. None of these subjects was found to carry any of the mutations. As for other inherited or acquired (antiphospholipid antibodies) thrombophilic risk factors, only the proband of family 4 was found to carry the factor V Leiden mutation. Of the 8 mutations identified, only the G→A transition at nucleotide 9788 and the nonsense mutation at the R132 residue have been previously reported.<sup>4-6</sup> The G→A transition 14 bp before the end

**Table 1. Clinical and biochemical characteristics of the AT-deficient subjects.**

	Sex	Age	Age at 1 <sup>st</sup> event	Type of thrombosis	Recurrence	AT activity (80-120%)	AT antigen (22-50 mg/dL)
Family 1	F	29	27	Proximal DVT	No	68%	19.3
Family 2	F	32	24	Proximal DVT	No	53%	16.9
Family 3	M	34	33	Bilateral proximal DVT and PE	No	54 %	18.0
Family 4	F	27	26	Proximal DVT and PE	No	42%	14.6
Family 5	M	53	49	Proximal DVT	49 yrs PE	60%	17.5
Family 6	F	35	22	Proximal DVT and PE	31 yrs PE	51 %	15.8
Family 7	F	35	—	—	—	49%	16.3
Family 8	F	32	32	Bilateral proximal DVT	No	64%	17.2

Normal values of AT activity and antigen are shown in parentheses. M: male; F: female; DVT: deep vein thrombosis; PE: pulmonary embolism.

**Table 2. Mutations in the AT gene identified in the AT-deficient subjects.**

N	Family	Mutation	New	Location	Type	Effect
1	4	2371T→G	Yes	Exon 1	Nonsense	Y-31X
2	6	2430insTT	Yes	Exon 2	Ins. Out of frame	aa. -2 prem. stop
3	7	L81delC	Yes	Exon 2	Del. Out of fra	aa. 81 prem. stop
4	5	5390→T	No	Exon 3a	Nonsense	R132X
5	1	5524G→T*	Yes	Exon 3a	Missense/splice site mutation	K176N/skipping of the exon 3a
6	2	7672C→T	Yes	Exon 4	Missense	P321S
7	8	9788G→A	No	Intron 4	New acceptor splice site	4 extra aa
8	3	9835T→A	Yes	Exon 5	Missense	V364D

\*Although the 5524G→T transversion causes the K176N missense mutation, the alteration of mRNA splicing is likely to have a greater effect; aa: amino acid.

of intron 4 leads to the formation of a new acceptor splice site.<sup>5</sup> Among the previously unreported mutations that we found, 4 are likely to give rise to null alleles: the Y-31 nonsense mutation, the TT insertion at L-12 and R164, the C deletion at L81, and the T→G transversion at K176. The deletion at L81 in exon 2 (delC) leads to a change of the corresponding amino acid and to the formation of a premature stop codon (CCC-CTG-AGT→CCC-TGA-GTA). The heterozygous insertion at L-12 found in exon 2 (T2430insTT) is predicted to change all corresponding amino acids encoded by the subsequent portion of the exon 2 leading to a premature stop codon within the exon 2 at position -2. Although the K176T→G transversion causes a lysine to asparagine substitution, this occurs at the invariant AG of the exon at the acceptor site and is likely to affect correct mRNA splicing as in the related AAG to AAA mutation.<sup>7</sup>

In family 6 the L81delC occurred within a codon in which two different small deletions (2706delT and 2705-6delCT) have been previously reported.<sup>8,9</sup> This codon is bridged by a homonucleotide tract (CCCC), a well-known recognized hot spot consensus sequence for point mutations.<sup>10</sup> The other 2 unrecognized mutations were missense substitutions, which occurred in exon 4 (P321S) and in exon 5 (V364D). The P321 is located in the turn between strand 2B and strand 3B and is a highly conserved residue among serpins. The V364 residue is located in the  $\beta$ -sheet A, at the bottom of strand 5A, and, in serpins, may be occupied by valine or leucine, all non-polar, aliphatic amino acids. In contrast, aspartic acid is a charged amino acid, which disrupts secondary structures, the H-bonding side chain competing directly with backbone H-bonds. Thus, it is conceivable that both substitutions impair correct AT folding and, in turn, structural integrity. The number of different mutations found in this mutation screen of only 8 families confirms the marked genetic heterogeneity in this group of patients and also suggests that the AT gene is hypermutable, most mutations arising randomly and often being responsible for truncated proteins.

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Thrombosis

### High factor VIII plasma levels as a risk factor for venous thrombosis: no evidence of inheritance from a family cohort study

We assessed inheritance of high factor VIII:C (FVIII:C  $\geq 150\%$ ) levels in 52 probands carrying factor V Leiden and 176 first-degree relatives. Although age-adjusted FVIII:C levels aggregated within families, relatives of probands with FVIII:C levels  $\geq 150\%$  and  $< 150\%$  were comparable regarding median FVIII:C levels, cumulative distribution curves, and annual incidence of venous thromboembolism. Aggregation of high FVIII:C levels could not be explained by inheritance and was not predictable from FVIII:C levels in probands.

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High FVIII:C levels have been recognized as a risk factor for venous thromboembolism (VTE). Only little is known about their inheritability. Polymorphisms have not been identified. Differences in FVIII:C levels related to ABO blood groups support a genetic contribution.<sup>1</sup> In a female twin study this contribution was 61%.<sup>2</sup>

We analyzed data from a previous family cohort study, designed to estimate the absolute risk of VTE in factor V Leiden carriers.<sup>3</sup> Consecutive patients with VTE and factor V Leiden (probands) and their first-degree relatives older than 15 years were enrolled.

Genetic effects on FVIII:C levels were assessed by calculation of Pearson correlations between residuals (i.e. differences between observed levels and expected levels as esti-