De novo mutations in antithrombin deficiency: high frequency and heterogeneous mechanisms

More than 78 *de novo* mutations (DNM) arise in the human genome per generation.[1] Most DNM are single-nucleotide variants (SNV), but small insertions or deletions (INDEL) and structural variants (SV) can also occur *de novo*.[1] This mutation rate may be elevated in certain individuals or concentrated in specific genomic regions due to risk factors such as advanced paternal age or particular genomic architectures.[1]

While neutral or beneficial mutations may drive human evolution, DNM affecting essential genes can disrupt biological systems and lead to diseases.[2] Identifying and characterizing pathogenic DNM can help elucidate disease mechanisms and highlight genomic regions prone to mutagenesis. This information has important diagnostic and clinical implications for patients and their families. In the context of hemostasis, studies on DNM are scarce and typically limited to case reports, especially in hemophilia.[3]

Antithrombin deficiency (ATD) is a rare autosomal dominant disorder caused by variants in the *SERPINC1* gene that significantly increase the risk of thrombosis due to the key anticoagulant role of this serpin.[4] There are two main types of ATD: type I (with reduced antigen levels) are rare and often present with a severe clinical phenotype; type II (with normal antigen levels but impaired function) are more common and typically have milder thrombotic consequences, especially in cases involving impaired heparin binding (type II Heparin Binding Site [HBS] deficiency).[4]

We studied 433 consecutive, unrelated individuals with ATD recruited over 26 years (1998-2024). All participants gave informed consent to participate in this study, which was approved by the Institutional Review Board of the Hospital Universitario Morales Meseguer, Murcia, Spain, and was conducted in accordance with the principles of the Declaration of Helsinki of 1964 and its subsequent amendments. Functional and biochemical analyses performed to characterize the disorder included anti-FXa and anti-FIIa functional assays, determination of antigen levels, biochemical evaluation of plasma AT by western blot analysis, evaluation of heparin affinity, and, in some cases, recombinant expression.[5] Molecular analysis of *SERPINC1* found 158 different variants (112 SNV, 25 small INDEL, and 21 SV) in 347 individuals. Next, we identified 210 patients with *SERPINC1* variants who had family history data and/or parental samples available. In 11 of the 210 screened cases (5.2%), 10 different *SERPINC1* variants were identified despite the fact that ATD was discarded in the parents, whose paternity/maternity was confirmed by genotyping 16 short tandem repeats. These findings indicate a *de novo* origin for these variants (Figure 1).

Remarkably, most cases with DNM (10/11; i.e., all except P11) presented a severe clinical phenotype, including idiopathic or early-onset thrombosis (median age at first event: 18 years; range: 3 months-36 years) and recurrent events in 50%. Median AT activity was 48%. Eight patients had type I, and three had type II Pleiotropic Effect deficiencies. None had Reactive Site or HBS deficiencies (Table 1).

Eight patients had *de novo* SNV scattered throughout *SERPINC1*, with no regional clustering. Transitions were more common than transversions (7:1), and none occurred at CpG dinucleotides (Figure 2). Six variants had been previously reported in other ATD cohorts, supporting multiple independent mutational events. Notably, 2 unrelated individuals (P8 and P10) had the same DNM: c.394C>T, p.Glu132*.

One patient carried a novel dinucleotide insertion c.1318_1319insTT leading to a frameshift and premature stop codon in the C-terminus of AT (Table 1). Two patients had gross *de novo* deletions involving *SERPINC1*. The length and characteristics of these deletions have been previously described in detail by our group.[6] Briefly, in P5, the deletion encompassed the entire gene and covered 29 additional loci. In P7, the deletion included exon 1 and two neighboring genes (Figure 2). In both cases, nanopore sequencing revealed repetitive elements flanking the breakpoints (L1PA2

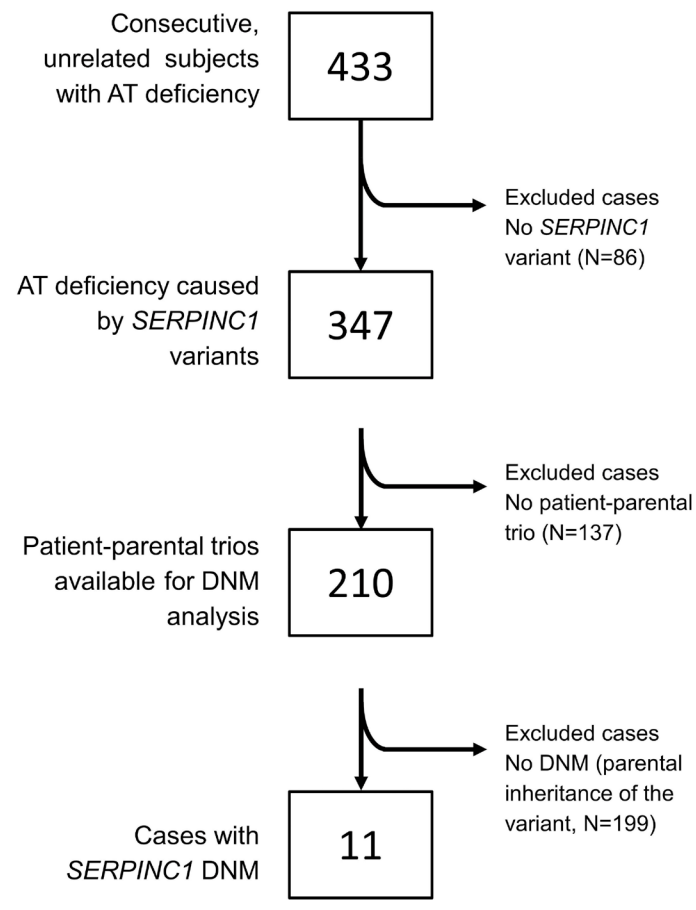


Figure 1. Flow chart of cases with *de novo* mutations in *SERPINC1* identified in our cohort of patients with antithrombin deficiency.

Table 1. Characteristics of cases with antithrombin deficiency caused by a *SERPINC1 de novo* mutation.

	Sex	Age years	Thrombosis (age at 1st event)	Recurrent VTE	AT Ac %†	AT Ag %†	Deficiency	Variant	Previously described‡ HGMD ID	Mutated allele
P1	Male	54	DVT (28 yr)	No	52	78%	IIPE	c.1201C>T p.His401Tyr	CM063127	Paternal
	F	82	No	-	112	102	-	-	-	-
	M	80	No	-	111	105	-	-	-	-
P2	Male	26	DVT (15 yr)	Yes	26	47	I	c.334C>T p.Pro112Leu	New	Paternal
	F	56	No	-	86	92	-	-	-	-
	M	53	No	-	83	89	-	-	-	-
P3	Male	28	DVT, pulmonary embolism (14 yr)	No	56	66	I	c.1141T>C p.Ser381Pro	CM920112	Maternal
	F	58	No	-	101	103	-	-	-	-
	M	53	No	-	99	106	-	-	-	-
P4	Female	33	DVT (19 yr)	Yes	44	71	IIPE	c.1317C>A p.Pro439Thr	CM921005	ND
	F	65	No	-	130	120	-	-	-	-
	M	63	No	-	114	107	-	-	-	-
P5	Male	24	DVT, pulmonary embolism, CVT	Yes	60	46	I	*SERPINC1* deletion (2MB)	New	Paternal
	F	63	No	-	91	98	-	-	-	-
	M	59	No	-	101	100	-	-	-	-
P6	Male	54	DVT (36 yr)	No	60	77	IIPE	c.817A>G p.Lys273Glu	CM104636	ND
	F	78	No	-	98	101	-	-	-	-
	M	75	No	-	97	104	-	-	-	-
P7	Male	2	CVT (3 weeks)	No	29	30	I	Exon 1 Deletion (19KB)	New	Maternal
	F	39	No	-	92	102	-	-	-	-
	M	36	No	-	75	90	-	-	-	-
P8	Female	45	DVT (22 yr)	Yes	50	51	I	c.394C>T p.Gln132*	CM992137	ND
	F	85	No	-	102	105	-	-	-	-
	M	74	No	-	96	98	-	-	-	-
P9	Female	17	Pulmonary embolism (14 yr)	No	59	60	I	c.1318_1319ins TT p.Leu441Ser fs*5	New	ND
	F	48	No	-	91	98	-	-	-	-
	M	56	No	-	91	107	-	-	-	-
P10	Female	20	DVT (18 yr)	Yes	45	51	I	c.394C>T p.Gln132*	CM992137	ND
	F	61	No	-	92	98	-	-	-	-
	M	59	No	-	117	105	-	-	-	-
P11	Female	33	No	-	43	48	I	c.685C>T p.Arg229*	CM920111	ND
	F	NA	No	-	107	110	-	-	-	-
	M	56	No	-	115	120	-	-	-	-

Continued on following page.

[†]Antithrombin activity and antigen are expressed as a percentage of reference plasma prepared from a pool of 100 healthy blood donors. [‡]Gene variants identified in other cohorts of patients with antithrombin deficiency. Previously described variants are indicated by the mutation code in the Human Gene Mutation Database (HGMD; https://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1). Ac: antithrombin activity (anti-FXa); AT: antithrombin; AT Ag: AT antigen; CVT: cerebral venous thrombosis; DVT: deep venous thrombosis; F: father; M: mother; ND: not determined; PE: pleiotropic effect; VTE: venous thromboembolism; yr: years.

and A-rich, respectively).

The low AT activity observed in DNM carriers (Table 1) argued against a somatic mosaicism as the underlying mechanism for the emergence of these variants. Evaluation of the electropherograms of the *de novo* SNV and INDEL also supported a germline origin in the probands. However, since Sanger sequencing lacks the sensitivity to detect and quantify low-level mosaicism or confirm a true germline DNM, deep sequencing by nanopore was performed in patients carrying these DNV SNV/INDEL. For each case, over 50,000 reads covering the mutation were analyzed, showing an approximately equal distribution of mutant and wild-type alleles in all probands, consistent with a germline origin. The same approach was applied to evaluate potential mosaicism in the parents. In all instances, the presence of the variant allele in parental samples was <1% of total reads (<0.1% in 5 cases).

We attempted to determine the paternal or maternal origin of the mutant allele for all carriers of SNV or the small insertion DNM by analyzing *SERPINC1* intragenic haplotypes in each trio using nanopore sequencing of long-range PCR products covering the gene.[7] However, likely due to the low genetic variability of *SERPINC1*, we were only able to identify the allele carrying the DNM (paternal or maternal) in 5 cases. Three DNM had a paternal origin (60%), and two maternal (40%) (Table 1).

To date, 546 different *SERPINC1* variants have been reported (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1) but only 10 (9 SNV and one small deletion) have been previously described as DNM causing ATD (Figure 2). Here, we provide the most comprehensive analysis to date of *SERPINC1* DNM in one of the largest ATD cohorts. Our results reveal a relatively high proportion of disease-causing DNM in *SERPINC1*, accounting for up to 5.2% of all cases screened. However, these results may only show the tip of the iceberg, and the true prevalence of DNM in *SERPINC1* might be underestimated. The severe clinical phenotype observed in most individuals with *SERPINC1* DNM, both in our cohort and in previously reported cases, indicates a likely diagnostic selection bias. Indeed, all DNM carriers had severe type I or type II Pleiotropic Effect defects, often presenting with early and/or recurrent thrombosis. Milder cases, such as those with type II RS, or especially type II HBS variants, may have been under-represented and missed from our study, as these typically have a lower thrombotic risk, delayed onset, or non-typical manifestations (e.g., arterial thrombosis), and often lack a family history of thrombosis,[4] features that commonly lead to exclusion from thrombophilia screening.[8]

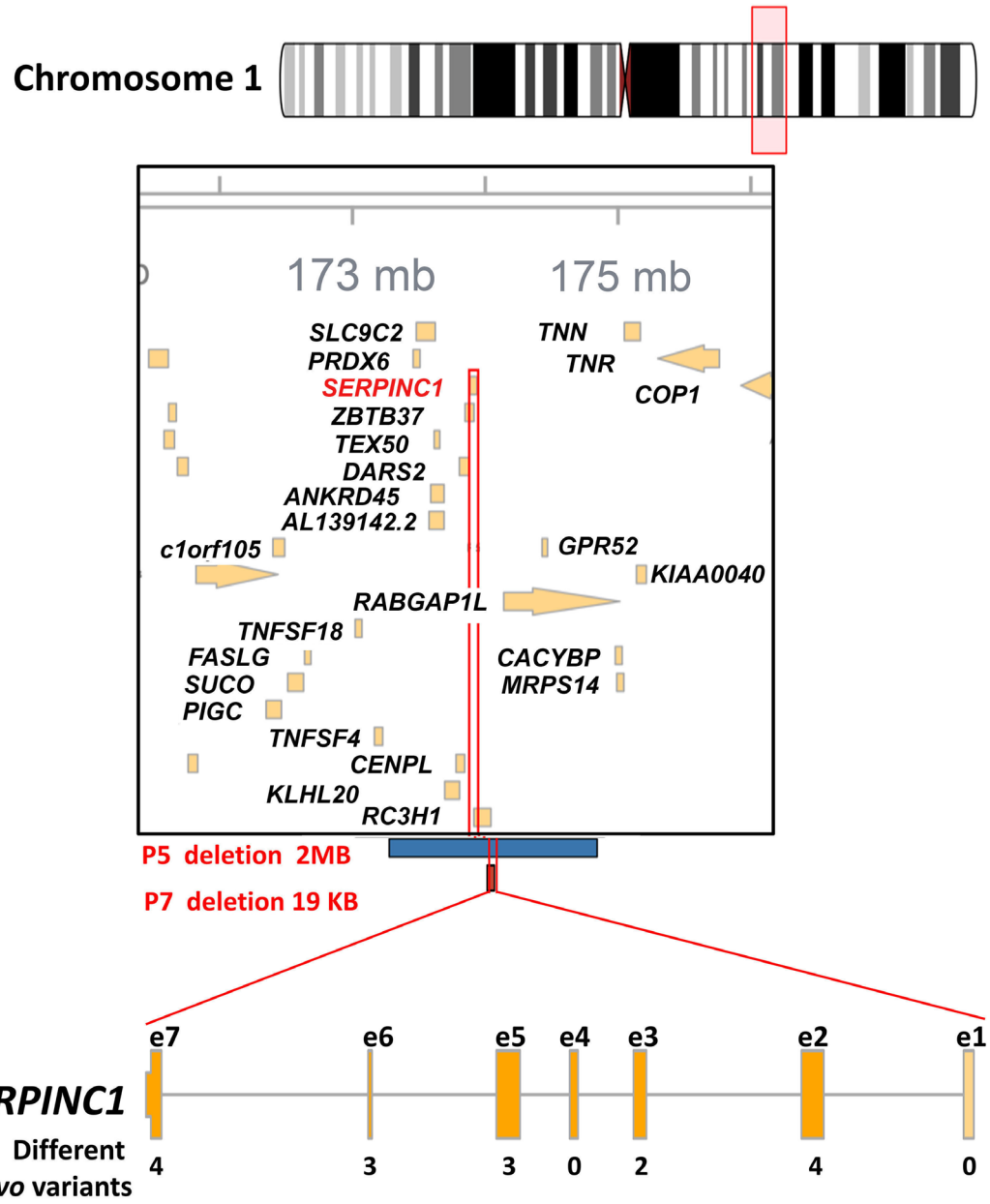
As in other disorders caused by DNM, most *SERPINC1* DNM are SNV. However, the expected enrichment of C>T transitions at CpG sites, attributed to higher methylation and spontaneous deamination in the male germline,[9] was not observed in *SERPINC1*, although these findings may be attributable to chance due to the small sample size and should be validated in further studies.

Regarding genomic distribution, *de novo* SNV in *SERPINC1* show some clustering in exons 6 and, particularly, exon 7, where 4 DNM were identified within an 18bp region. This finding aligns with prior evidence suggesting exon 7 is a hotspot due to repetitive DNA sequences.[10] However, the majority of *SERPINC1* DNM are scattered across the gene without clear regional susceptibility. An exception is c.394C>T, p.Glu132*, a recurrent mutation site, as it was found as a DNM in 2 unrelated cases in our cohort and was previously reported in a Japanese patient with type I deficiency.[11]

Notably, we report the first cases of gross DNM causing ATD, both deletions involving repetitive elements, which are well known to mediate genomic rearrangements.[12] *SERPINC1* is flanked and interspersed with a high proportion of repetitive elements, some of which have been implicated in ATD through SV.[6]

Finally, our study explores the origin of the DNM in ATD with the following considerations. 1) Potential germline origin: deep sequencing found <1% of somatic mosaicism for SNV and INDEL in both patients and parents, supporting germline origin. However, recent studies showed that apparent DNM occasionally originate from undetected parental mosaicism, sometimes with variant allele frequencies <1%.[13] Moreover, gonadal mosaicism cannot be ruled out by peripheral blood testing. In addition, the absence of a detectable mosaic fraction does not exclude a very early post-zygotic origin, particularly for SV, due to the instability of the early zygotic genome associated with aneuploidy and gross rearrangements,[14] which could still manifest as a constitutional pattern. 2) Paternal bias: since 1947, it has been hypothesized that the male germline may be more mutagenic than the female germline.[15] However, whole-genome analyses of parent-offspring trios have revealed substantial inter-family variation.[1] In our study, 40% of the DNM occurred on the maternal allele, suggesting that, for *SERPINC1*, there is no strong male bias. This finding is clinically relevant, as maternal DNM carry a risk of recurrence in subsequent pregnancies.[1] 3) Parental age: increasing paternal age is associated with higher DNM rates due to replication errors during the cell divisions required for continuous sperm production.[16] In modern societies,

where delayed parenthood is increasingly common, this trend raises concern about the rising incidence of *de novo* genetic disorders.[16] In our study, the average age of fathers (32.2 years) and mothers (29.8 years) was not markedly high, suggesting parental age has a minimal impact on the generation of *SERPINC1* DNM. The small sample size



Exon	Nucleotide	Aminoacid	Patient	HGMD ID	Reference *de novo* variant
2	c.259A>G	N87D		CM1714819	Kamijima *et al*. (2018)[17]
	c.279_282del	F94I fs*19		CD041608	Orlando *et al*. (2012)[18]
	c.334C>T	P112L	P2	New	-
	c.394C>T	Q132*	P8 &P10	CM992137	Corral *el al*. (2004)[19]
3	c.624G>A	K208K		CS920728	Berg *et al*. (1992)[20]
	c.685C>T	R229*	P11	CM920111	Not mentioned as a *de novo* variant
5	c.817A>G	K273E	P6	CM104636	Martínez *et al*. (2010)[5]
	c.907G>T	E303*		CM990200	Tarantino *et al*. (1999)[21]
	c.1141T>C	SA381P	P3	CM920112	Not mentioned as a *de novo* variant
6	c.1171C>T	R391*		CM930056	Orlando *et al*. (2012)[18]
	c.1196C>A	A399E		CM176655	Wang *et al*. (2017)[22]
	c.1201C>T	H401Y	P1	CM063127	Not mentioned as a *de novo* variant
7	c.1301T>C	F434S		CM910060	Lane *et al*. (1992)[23]
	c.1306G>A	A436T		CM890020	Arnaldi *et al*. (2001)[24]
	c.1317C>A	P439T	P4	CM921005	Not mentioned as a *de novo* variant
	c.1318_1319insTT	L441S fs*5	P9	New	-

Figure 2. Localization in *SERPINC1* of *de novo* mutations identified in this and other studies. This study (in red) and other studies (in black). The blue rectangle indicates the extension of the deletion.

strongly encourages evaluating both paternal bias and age in further studies.

In conclusion, we report a relatively high frequency of *SERPINC1* DNM causing ATD: 5.2%, a value that may be underestimated due to clinical selection bias. DNM in *SERPINC1* exhibit features distinct from those in other disorders with high DNM rates, including a notable proportion of SV and a lack of enrichment for C>T transitions at CpG sites. Our data suggest that most *SERPINC1* DNM originate during gametogenesis in parents of non-advanced age. These findings support systematic thrombophilia screening, including molecular analysis, in patients with ATD, regardless of family history.

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Disclosures

No conflicts of interest to disclose.

Contributions

PG-R, BM-B, MEM-B and JC designed and conducted the research, and analyzed data; CB-P, RC-R, JP, EN, ML-L, AM and RT conducted the research and analyzed data; AR-A, FV, MJB, MFL-F, BF-P, SA, JRG-P, VV and MLL provided patient data and performed data analysis. All authors wrote the article.

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Data-sharing statement

All data and results generated during this study are available from the corresponding author upon request. Researchers interested in accessing these materials may contact the corresponding author, who will provide them without undue restriction.

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