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

Pedro Garrido-Rodríguez,[1*] Belén de la Morena-Barrio,[1*] Carlos Bravo-Pérez,[1] Rosa Cifuentes-Riquelme,[1] José Padilla,[1] Esther Navarro,[1] María Llamas-López,[1] Antonia Miñano,[1] Raúl Teruel,[1] Agustín Rodríguez-Alen,[2] Francisco Velasco,[3] María J. Blanco,[4] María F. López-Fernández,[5] Beatriz Fernández-Pérez,[6] Susana Asenjo,[7] José R. González-Porras,[8] Vicente Vicente,[1] María L. Lozano,[1] María E. de la Morena-Barrio[1#], Javier Corral.[1#]

[1] Servicio de Hematología, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB, CIBERER-ISCIII, Murcia, Spain. [2] Hospital Universitario de Toledo, Spain. [3] Department of Medicine. University of Cordoba (UCO). Maimonides Biomedical Research Institute of Córdoba (IMIBIC). Córdoba, Spain. [4] Departamento de Hematología, Hospital Universitario La Paz, Madrid, Spain. [5] Servicio de Hematología, Complejo Hospitalario Universitario A Coruña, A Coruña, Spain. [6] Servicio de Hematología Hospital Francesc de Borja. Valencia, Spain. [7] Servicio de Hematología, Hospital Clínico San Carlos, Madrid, Spain. [8] Departamento de Hematología, Complejo Asistencial Universitario de Salamanca (CAUSA), Instituto de Investigación Biomédica de Salamanca (IBSAL), Universidad de Salamanca (USAL), 37007 Salamanca, Spain.

*** These authors contributed equally as co-first authors**

 # These authors contribute equally as co-senior authors

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Correspondence:

Javier Corral. Servicio de Hematología, Hospital Universitario Morales Meseguer, Centro Regional de Hemodonación, Universidad de Murcia, IMIB, CIBERER-ISCIII, Murcia, Spain; javier.corral@carm.es

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Author Contributions

P.G-R, B.M-B; M.E.M-B, and J.C: designed the research, conducted the research, analyzed data, and wrote the article. C.B-P, R.C-R, J.P, E.N, M.L-L, A.M, and R.T: conducted the research, analyzed data and wrote the article. A.R-A, F.V, M.J.B, M.F.L-F, B.F-P, S.A, J.R.G-P, V-V and ML.L: provided patient data, analyzed data and wrote the article.

Disclosures

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More than 78 *de novo* mutations (DNMs) arise in the human genome per generation.[1] Most DNMs are single-nucleotide variants (SNVs), but small insertions or deletions (INDELs), and structural variants (SVs) 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, DNMs affecting essential genes can disrupt biological systems and lead to diseases.[2] Identifying and characterizing pathogenic DNMs 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 DNMs 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] Two main types of ATD exist: 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 Hospital Universitario Morales Meseguer and was conducted in accordance with 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 SNVs, 25 small INDELs, and 21 SVs) in 347 individuals. Next, we identified 210 patients with *SERPINC1* variants who had a family history data and/or parental samples available. In 11 of the 210 screened cases (5.2%), 10 different *SERPINC1* variants were identified despite 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 DNMs (10/11, 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* SNVs 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, two 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 described in detail by our group previously.[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 and A-rich, respectively).

The low AT activity observed in DNMs 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* SNVs 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 SNVs/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).

The paternal or maternal origin of the mutant allele was determined for all carriers of SNVs 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 five 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 SNVs 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* DNMs in one of the largest ATD cohorts. Our results reveal a relatively high proportion of disease-causing DNMs 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* DNMs, 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 underrepresented 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 DNMs, most *SERPINC1* DNMs are SNVs. 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* SNVs in *SERPINC1* show some clustering in exons 6 and particularly exon 7, where 4 DNMs were identified within an 18bp region. This finding aligns with prior evidence suggesting exon 7 as a hotspot due to repetitive DNA sequences.[10.]However, the majority of *SERPINC1* DNMs 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 two unrelated cases in our cohort and previously reported in a Japanese patient with type I deficiency.[11]

Notably, we report the first cases of gross DNMs 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 SVs.[6]

Finally, our study dissects the origin of the DNMs in ATD with the following considerations:

1) Potential germline origin. Deep sequencing found <1% of somatic mosaicism for SNVs and INDEL in both patients and parents, supporting germline origin. However, recent studies showed that apparent DNMs occasionally originate from undetected parental mosaicism, sometimes with variant allele frequencies <1%.[13] Moreover gonadal mosaicism can't be discarded with peripheral blood testing. In addition, the absence of a detectable mosaic fraction does not exclude a very early post-zygotic origin, particularly for SVs 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 DNMs occurred on the maternal allele, suggesting that for *SERPINC1*, there is no strong male bias. This finding is clinically relevant, as maternal DNMs 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 limited impact of parental age in the generation of *SERPINC1* DNMs.

The small sample size strongly encourages evaluating both paternal bias and age in further studies.

In conclusion, we report a relatively high frequency of *SERPINC1* DNMs causing ATD: 5.2%, a value that may be underestimated due to clinical selection bias. DNMs in *SERPINC1* exhibit features distinct from those in other disorders with high DNM rates, including a notable proportion of SVs and a lack of enrichment for C>T transitions at CpG sites. Our data suggest that most *SERPINC1* DNMs 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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Tables

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

	Sex	Age	Thrombosis (Age 1st event)	Recurrent VTE	AT Ac (%)†	AT Ag (%)†	Deficiency	Variant	Previously described ‡ HGMD ID	Mutated allele
P1	Male	54	DVT (28y)	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 (15y)	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 (14y)	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 (19y)	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 (13y)	Yes	60	46	I	*SERPINC1* deletion (2MB)	New	Paternal
	F	63	No	--	91	98	--	--	--	--
	M	59	No	--	101	100	--	--	--	--
P6	Male	54	DVT (36y)	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 (22y)	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 (14y)	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 (18y)	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	--	No	--	107	110	--	--	--	--
	M	56	No	--	115	120	--	--	--	--

† Antithrombin activity and antigen are expressed as a percentage of reference plasma prepared from a pool of 100 healthy blood donors

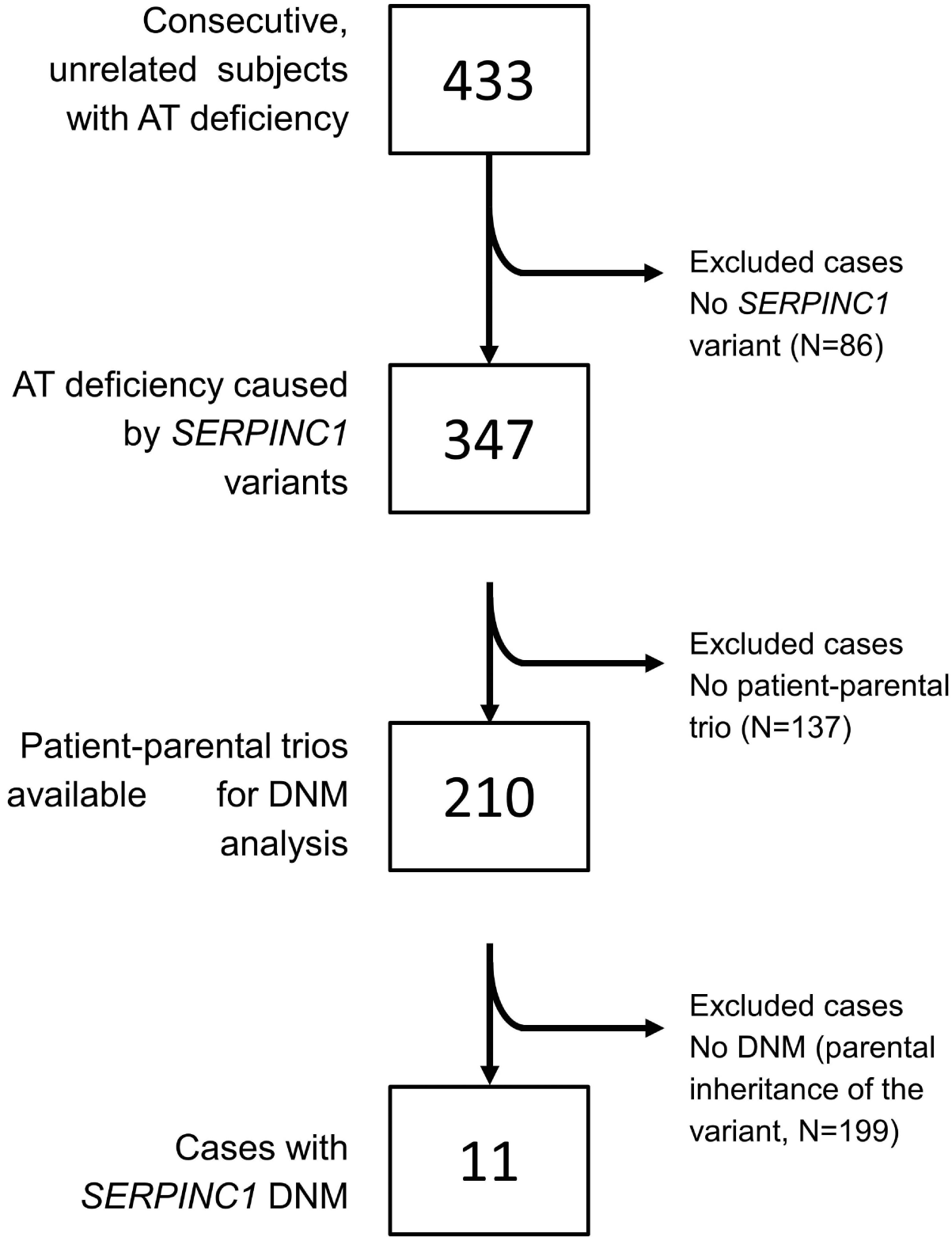
‡ Gene variants identified in other cohorts of patients with antithrombin deficiency are shown in bold. Previously described variants are indicated by the mutation code in the Human Gene Mutation Database (HGMD, https://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1).

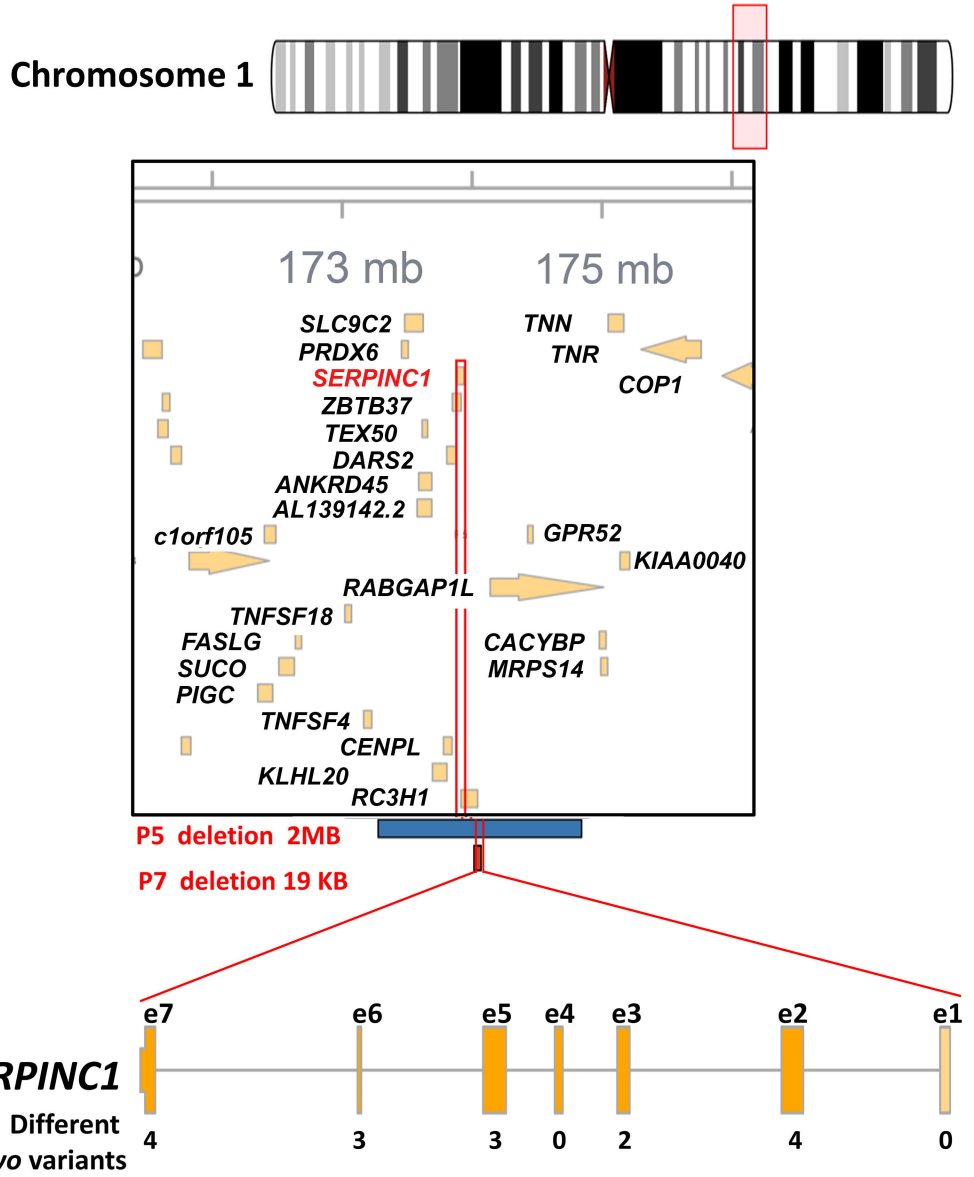
AT: Antithrombin. Ac: AT activity (anti-FXa). AT Ag: AT antigen. CVT: Cerebral venous thrombosis. DVT: Deep venous thrombosis. F: Father; M: Mother. ND: Not determined. PE: Pleiotropic effect

Figure legends

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

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


Consecutive, unrelated subjects with AT deficiency
433
Excluded cases
No *SERPINC1* variant (N=86)
AT deficiency caused by *SERPINC1* variants
347
Excluded cases
No patient-parental trio (N=137)
Patient-parental trios available for DNM analysis
210
Excluded cases
No DNM (parental inheritance of the variant, N=199)
Cases with *SERPINC1* DNM
11



Exon	Nucleotide	Aminoacid	Patient	HGMD ID	Reference *de novo* variant
2	c.259A>G	N87D		CM1714819	Kamijima et al. Int J Hematol 2018
	c.279_282del	F94I fs*19		CD041608	Orlando et al. Thromb Haemost 2012
	c.334C>T	P112L	P2	New	-
	c.394C>T	Q132*	P8 &P10	CM992137	Corral el al. Circulation 2004
3	c.624G>A	K208K		CS920728	Berg et al. Genomics 1992
	c.685C>T	R229*	P11	CM920111	No mentioned as a *de novo* variant
5	c.817A>G	K273E	P6	CM104636	Martínez et al. Haematologica 2010
	c.907G>T	E303*		CM990200	Tarantino et al. Am J Hematol 1999
	c.1141T>C	SA381P	P3	CM920112	No mentioned as a *de novo* variant
6	c.1171C>T	R391*		CM930056	Orlando et al. Thromb Haemost 2012
	c.1196C>A	A399E		CM176655	Wang et al. Br J Haematol 2017
	c.1201C>T	H401Y	P1	CM063127	No mentioned as a *de novo* variant
7	c.1301T>C	F434S		CM910060	Lane et al. J Clin Invest 1992
	c.1306G>A	A436T		CM890020	Arnaldi et al. Thromb Res 2001
	c.1317C>A	P439T	P4	CM921005	No mentioned as a *de novo* variant
	c.1318_1319insTT	L441S fs*5	P9	New	-