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Functional and clinical significance of novel *SERPINA1* variants on alpha-1 antitrypsin deficiency

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Abstract

Background Mutations in the *SERPINA1* gene can result in alpha-1 antitrypsin deficiency (AATD), which may be associated with lung or liver injury. Although the S and Z alleles account for over 95% of cases of AATD, a wide variety of rare variants have been linked to deficiency and dysfunction, while other variants are associated with normal alpha-1 antitrypsin (AAT) levels and activity. Here, we present the identification and characterization of thirteen rare *SERPINA1* variants discovered during the genetic diagnosis of AATD by the Progenika diagnostic network.

Methods The new variants were identified by sequencing the exons of *SERPINA1* gene in cases with discrepancies between AAT serum levels and initial genotyping. In order to determine their pathogenic impact, the variants were expressed in a cellular model and evaluated for AAT secretion, intracellular accumulation and elastase inhibitory activity. In addition, protein structural mapping of the variants and analysis of positioning and residue/atomic contacts were performed.

Results The in silico and functional in vitro analysis allowed us to classify these AAT variants as six deficient (p.Val234Glu, p.Val242_Pro243insLeu, p.Leu291Phe, p.Ala308Ser, p.Pro393Thr and p.Pro393Arg), one dysfunctional (p.Thr96Ile), three normal (p.Ser71Arg, p.Ala349Pro and p.Asp365Glu) and three null alleles (p.Gln33*, p.Gln285* and p.Leu310Phefs*14).

Conclusions Functional assays and protein structural information are useful tools in the characterization of novel variants of the *SERPINA1* gene. The newly characterized mutations expand the number of *SERPINA1* variants with proven pathogenic effects, facilitating the diagnoses of future cases of AATD.

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Keywords Alpha1 antitrypsin deficiency, Mutations, Diagnosis, SERPINA1

Introduction

Alpha-1 antitrypsin deficiency (AATD) is an autosomal codominant genetic disorder characterized by insufficient levels of the protein alpha-1 antitrypsin (AAT) in serum, which protects lung tissues from enzyme-mediated proteolysis. The clinical manifestations of AATD include pulmonary emphysema due to a protease-antiprotease imbalance and/or liver disease due to the accumulation of AAT in hepatocytes [1]. AAT is encoded by the *SERPINA1* gene, with over 500 variants identified to date [2]. The most common alleles are referred as the M-alleles, which are associated with normal serum AAT levels. The most prevalent disease associated variants are Z (Glu342Lys) and S (Glu264Val), which significantly reduce serum AAT concentrations [3]. However, there are numerous rare, uncharacterized variants whose identification and analysis remain unexplored [4]. A systematic review of published studies identified numerous rare variants around the world, highlighting geographical differences in AATD variant patterns. The findings emphasize the need for comprehensive genetic testing to improve diagnosis, treatment, and outcomes for patients with AATD [5]. Identifying and characterizing new mutations is crucial for understanding the genetic diversity of this disease and its clinical impact, not only because it helps to provide more accurate diagnoses, but it also offers new insights into the molecular mechanisms underlying this condition [6].

The Progenika Diagnostic Network was established to aid in the diagnosis of AATD based on buccal swabs and dried blood spots, and had been implemented in numerous countries worldwide [7]. So far, this network has allowed the study of the distribution of known alleles and evaluation of the frequency and distribution of less common alleles associated with AATD [7, 8]. In this study, we have conducted an exhaustive analysis of the data obtained by the Progenika Diagnostic Network to identify and characterize thirteen rare AAT variants associated with AATD. Our aim was to provide a deeper understanding of the genetic diversity of AATD and its connection to clinical manifestations, thereby contributing to a more informed diagnostic process and supporting the clinical management of individuals with AATD.

Materials and methods

This was a secondary analysis from a study that evaluated the viability of the Progenika diagnostic genotyping system in six different countries by analyzing 30,827 samples from cases of suspected AATD [7].

New variants of *SERPINA1* gene were identified through the diagnostic procedures implemented by the Progenika diagnostic network [8]. The network enables rapid AATD diagnosis via direct genotyping of the 14 most frequent known mutations using buccal swabs or dried blood spots. Sequencing of *SERPINA1* gene was performed when discrepancies arose between AAT levels and the identified allelic variants, or upon the request of the attending clinician. Further details on *SERPINA1* sequencing are provided in the Supplementary file. Anonymized clinical data was entered into the Progenika web platform (https://grifolsalpha1test.com/) by the responsible physician. A limited set of variables were collected, including age, gender, smoking habits, liver and respiratory function parameters, and the reason for suspicion of AATD. A glossary of the terms and reference values used is provided in the Supplementary file.

In silico analysis of genetic variants

The Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) was used to investigate the population frequency of the newly identified variants. The pathogenic impact of the amino acid changes was assessed by several predictors including Sorting Intolerant From Tolerant (SIFT) and Polyphen-2 scores present in the Ensembl Variant Effect Predictor tool (VEP). (https://www.ensembl.org/Multi/Tools/VEP?db=core), and AlphaMissense (https://alphamissense.hegelab.org/). In addition, Varsome (https://varsome.com/) was consulted to classify the variants according to the ACMG/AMP guidelines. Finally, the variants were mapped in the protein 3D structure with Open-Source PyMOL Molecular Graphics System 2.5.0 (Schrödinger). Atomic distances were assessed with the Pymol Measurement Wizard. Homology structural models for variants were built with the SwissModel server [9] using the 3NE4 structure [10] as a template.

Site-directed mutagenesis

Expression vector encoding M1(Val213) AAT based on plasmid pCMV6 (OriGene) was used for in vitro expression of the newly identified variants. The Z variant and the new variants were introduced by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions and the primers described in Table S1.

Cell culture and transfection

HEK293T cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich). Transient transfections were performed with Lipofectamine 2000 reagent

(Thermo Fisher) and plasmids encoding the AAT variants in serum-free OptiMEM cultured medium (Gibco/Thermo Fisher). Cells and conditioned culture media were harvested 48 h after transfection and used for subsequent experiments.

Periodic Acid–Schiff (PAS) staining and immunofluorescence

Transfected HEK293T cells were fixed with 4% paraformaldehyde (PFA) and subjected to PAS and hematoxylin staining or immunostaining with anti-human AAT polymer 2C1.

Western blot analysis

Cell media of transfected HEK293T cells was collected and the cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with cOmplete Protease Inhibitor Cocktail (Roche). Cell media and cell lysates were analysed by PAGE, as described in Supplementary file.

Elastase inhibition assay

Cell media of transfected HEK293T cells was collected and AAT concentration was measured by immune nephelometry. Equal amounts of protein were used for the elastase inhibition assays, as previously described [11].

Results

Identification of new *SERPINA1* variants

Twelve individuals, whose clinical characteristics are summarized in Table 1, harboured the thirteen rare variants that have been characterized in this study (case 2 presented two variants). Eight variants were not previously described and five variants, although described, were not previously functionally characterized. The variants comprised nine missense, one non-frameshift insertion, and three nonsense mutations (Table 2).

Nonsense mutations introduce premature stop codons, leading to unstable mRNA or non-functional proteins that are degraded. The pathogenic effects of missense and non-frameshift variants are harder to determine. Consequently, we subjected the ten identified missense and non-frameshift variants to functional characterization in terms of intracellular accumulation, extracellular secretion and elastase inhibition activity. The in vitro effect, the in silico characterization and the relationship with the patient's clinical information, are discussed below for each variant.

p.Gln33Ter (Q0 Terres Del Ebre)

The proband was a 52-year-old female smoker (14 pack-years) who consulted for productive cough. Respiratory function, chest X-ray and liver function tests were normal, but low AAT serum levels were detected (50 mg/dL). The A1AT genotyping test showed a heterozygous S variant of *SERPINA1.* Complete sequencing revealed an additional c.97 C > T (p.Gln33Ter) variant.

The p.Gln33Ter is the result of a base-pair substitution in codon 33, causing a premature stop codon. This variant is likely to affect mRNA processing or truncated protein stability and was deemed a null allele; therefore, we classified it as pathogenic in accordance with ACMG/AMP guidelines [12].

p.Ser71Arg (S Roubaix) and p.Leu310PhefsTer14 (Q0 Olórdola)

The variants p.Ser71Arg and p.Leu310PhefsTer14 were identified in a 34-year-old male with reduced AAT serum levels (66 mg/dL) and a family history of AATD. He was a non-smoker with no pathological history and without respiratory symptoms. Respiratory and liver function laboratory tests were normal. The subject underwent the A1AT genotyping test without observing any of the 14 mutations evaluated. Complete sequencing of the *SERPINA1* gene showed two variants: c.211 A > C

Table 1 Clinical information of the patients carrying the new *SERPINA1* variants

Case	Genotype	Age (years)	Sex	AAT (mg/dl)	Smoking	FEV1 (%)	Liver disease
1	S / c.97 C > T (p.Gln33Ter)	52	female	50	yes	100	no
2	c.211 A > C (p.Ser71Arg) / c.930del (p.Leu310Phefs*14)	34	male	66	no	95	no
3	Z / c.287 C > T (p.Thr96Ile)	75	female	59	no	91	no
4	M / c.701T > A (p.Val234Glu)	29	female	50	no	98	yes
5	M / c.725_727dup (p.Val242_Pro243insLeu)	74	male	58	yes	43	no
6	M / c.853 C > T (p.Gln285Ter)	48	male	73	yes	97	no
7	M / c.871 C > T (p.Leu291Phe)	69	male	54	yes	52	no
8	S / c.922G > T (p.Ala308Ser)	72	male	43	yes	41	no
9	M / c.1045G > C (p.Ala349Pro)	71	female	117	yes	54	no
10	M / c.1095 C > G (p.Asp365Glu)	24	male	60/142	no	NA	no
11	Z / c.1177 C > A (p.Pro393Thr)	53	female	40	yes	121	no
12	c.1178 C > G (p.Pro393Arg) / c.1178 C > G (p.Pro393Arg)	60	female	60	yes	108	no

*Abbreviations**AAT* alpha-1 antitrypsin, *FEV1* forced expiration volume in 1 s, *NA* not available

Table 2 Description of novel *SERPINA1* variants, predicted impact and functional effects (compared to M allele)

Case	Variant name	Nucleotide change (HGVS)	Amino acid change (HGVS)	Amino acid change (mature protein)	Variant Type	Exon	dbSNP	Frequency	SIFT	PolyPhen	Alphamissense	Varsome	In vitro effect	Allele and variant classification [1]
1	Q0 Terres del Ebre	c.97 C > T	p.(Gln33*)	(Gln9*)	Nonsense	2	NA	0	NA	NA	NA	Likely pathogenic	NA	Null (pathogenic)
2	S Roubaix	c.211 A > C	p.(Ser71Arg)	(Ser47Arg)	Missense	2	rs11575873	9.12e-5	Tolerated	Benign	Likely benign	Likely benign	- No polymerization - Normal secretion - Normal anti-elastase activity	Normal (likely benign)
	Q0 Olórdola	c.930del	p.(Leu310Phefs*14)	(Leu286Phefs*14)	Nonsense	4	NA	0	NA	NA	NA	Likely pathogenic	NA	Null (pathogenic)
3	Pi Getafe	c.287 C > T	p.(Thr96Ile)	(Thr72Ile)	Missense	2	NA	0	Deleterious	Probably damaging	Likely pathogenic	Uncertain significance	- No polymerization - Normal secretion - Reduced anti-elastase activity	Dysfunctional (likely pathogenic)
4	Pi Viladecans	c.701T > A	p.(Val234Glu)	(Val210Glu)	Missense	3	rs746197812	3.56e-5	Deleterious	Probably damaging	Likely pathogenic	Uncertain significance	- Polymerization - Impaired secretion	Deficient (likely pathogenic)
5	Pi Cazorla	c.725_727dup	p.(Val242_Pro243insLeu)	(Val218_Pro219insLeu)	In-framed InDel	3	NA	0	NA	NA	NA	Uncertain significance	- Polymerization - Impaired secretion	Deficient (likely pathogenic)
6	Q0 Donosti	c.853 C > T	p.(Gln285*)	(Gln261*)	Nonsense	3	rs1480528329	1.86e-6	NA	NA	NA	Likely pathogenic	NA	Null (pathogenic)
7	Pi São Mateus do Sul	c.871 C > T	p.(Leu291Phe)	(Leu267Phe)	Missense	3	rs1443274556	1.24e-6	Deleterious	Probably damaging	Likely benign	Uncertain significance	- Polymerization - Impaired secretion	Deficient (likely pathogenic)
8	M1 Lyon	c.922G > T	p.(Ala308Ser)	(Ala284Ser)	Missense	4	rs141620200	3.20e-3	Deleterious	Benign	Likely benign	Likely benign	- Polymerization - Normal secretion	Mild Deficient (likely pathogenic)

Table 2 (continued)

Case	Variant name	Nucleotide change (HGVS)	Amino acid change (HGVS)	Amino acid change (mature protein)	Variant Type	Exon	dbSNP	Frequency	SIFT	PolyPhen	Alphamissense	Varsome	In vitro effect	Allele and variant classification [1]
9	Pi Almirante Tamandaré	c.1045G > C	p.(Ala349Pro)	(Ala325Pro)	Missense	4	rs376024688	1.24e-6	Tolerated	Benign	Likely benign	Likely benign	- No polymerization - Normal secretion - Normal anti-elastase activity	Normal (likely benign)
10	Pi Istambul	c.1095 C > G	p.(Asp365Glu)	(Asp341Glu)	Missense	5	rs201774333	4.27e-5	Deleterious	Benign	Likely pathogenic	Uncertain significance	- No polymerization - Normal secretion - Normal anti-elastase activity	Normal (likely benign)
11	Pi Complutense	c.1177 C > A	p.(Pro393Thr)	(Pro369Thr)	Missense	5	rs61761869	6.20e-7	Deleterious	Probably damaging	Ambiguous	Likely pathogenic	- Polymerization - Impaired secretion	Deficient (pathogenic)
12	Pi Ponferrada	c.1178 C > G	p.(Pro393Arg)	(Pro369Arg)	Missense	5	rs199422209	2.48e-5	Deleterious	Probably damaging	Likely pathogenic	Likely pathogenic	- Polymerization - Impaired secretion	Deficient (pathogenic)

[1] in accordance with ACMG/AMP guidelines [12]

The reference sequence used for the *SERPINA1* transcript annotation is National Center for Biotechnology Information NM_001127701, and the reference sequence for the *SERPINA1* protein is UniProt P01009. Variants are sorted by gene location

*Abbreviations**NA* not available, *HGVS* human genome variation society, *dbSNP* database single nucleotide poplymorphism, *SIFT* sorting intolerant from tolerant

(p.Ser71Arg) and c.930del (p.Leu310Phefs*14). Familial co-segregation analysis demonstrated that both variants were in *trans*.

The p.Leu310PhefsTer14 is the result of a 1 base-pair deletion in codon 310, causing a frameshift and a premature termination codon at position 323. This variant is likely to affect the mRNA processing or truncated protein stability and was deemed a null allele; therefore, we classified it as as pathogenic.

The p.Ser71Arg variant was predicted as benign by three pathogenicity prediction algorithms (SIFT, Polyphen-2 and Alphamissense) and classified as likely benign by Varsome. The structural mapping showed that p.Ser71Arg involves a hydroxyl-to-charged group substitution. However, the fact that this side chain is oriented outwards to the solvent without any contact with other residues indicate this change could be tolerated without any remarkable biochemical or clinical phenotype (Fig. 1). Regarding functional studies, cells expressing variant p.Ser71Arg were negative for PAS staining (Fig. 2A) and for AAT polymer 2C1 antibody (Fig. 2B), and did not show intracellular accumulation of polymerogenic AAT in the insoluble fraction when analysed by non-denaturing western blot (Fig. 3A). In addition, secretion efficiency of AAT was analysed by western blot in the culture media of transfected cells and the variant demonstrated efficient secretion into the medium (Fig. 3B). Finally, we performed an elastase inhibition assay in the cell media of secreted variants. The variant inhibited elastase activity by 85%, similar to the inhibitory activity of the WT AAT (90%) (Fig. 4). To sum up, p.Ser71Arg variant showed no AAT polymerization, normal secretion and normal anti-elastase activity when studied in vitro; therefore, we have classified it as a likely benign variant and consider it to represent a normal AAT allele.

It is noteworthy that p.Ser71Arg variant has been previously described [13] and classified as likely benign, although no functional study was performed.

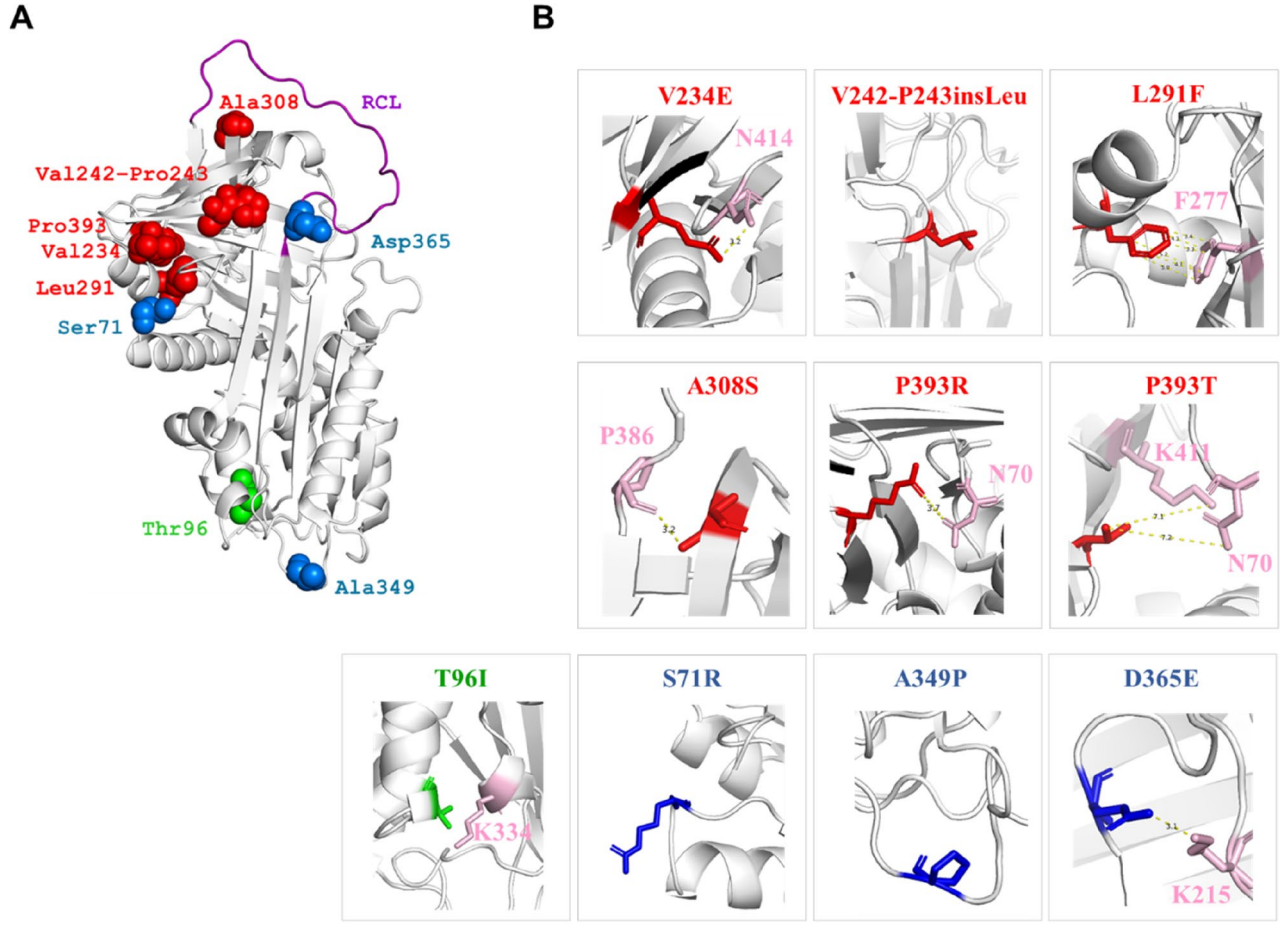


Fig. 1 Structural mapping of the new missense and non-frameshift AAT variants identified. **A** Localization in the AAT protein structure of the amino acid residues corresponding to each variant, highlighted in red (deficient variants), green (dysfunctional variant) or blue (normal variants). Residues are numbered according to the unprocessed, immature form of the protein. **B** Detail of the predicted structural changes produced by the amino acid substitutions of each variant, showing the new residue interactions (pink)

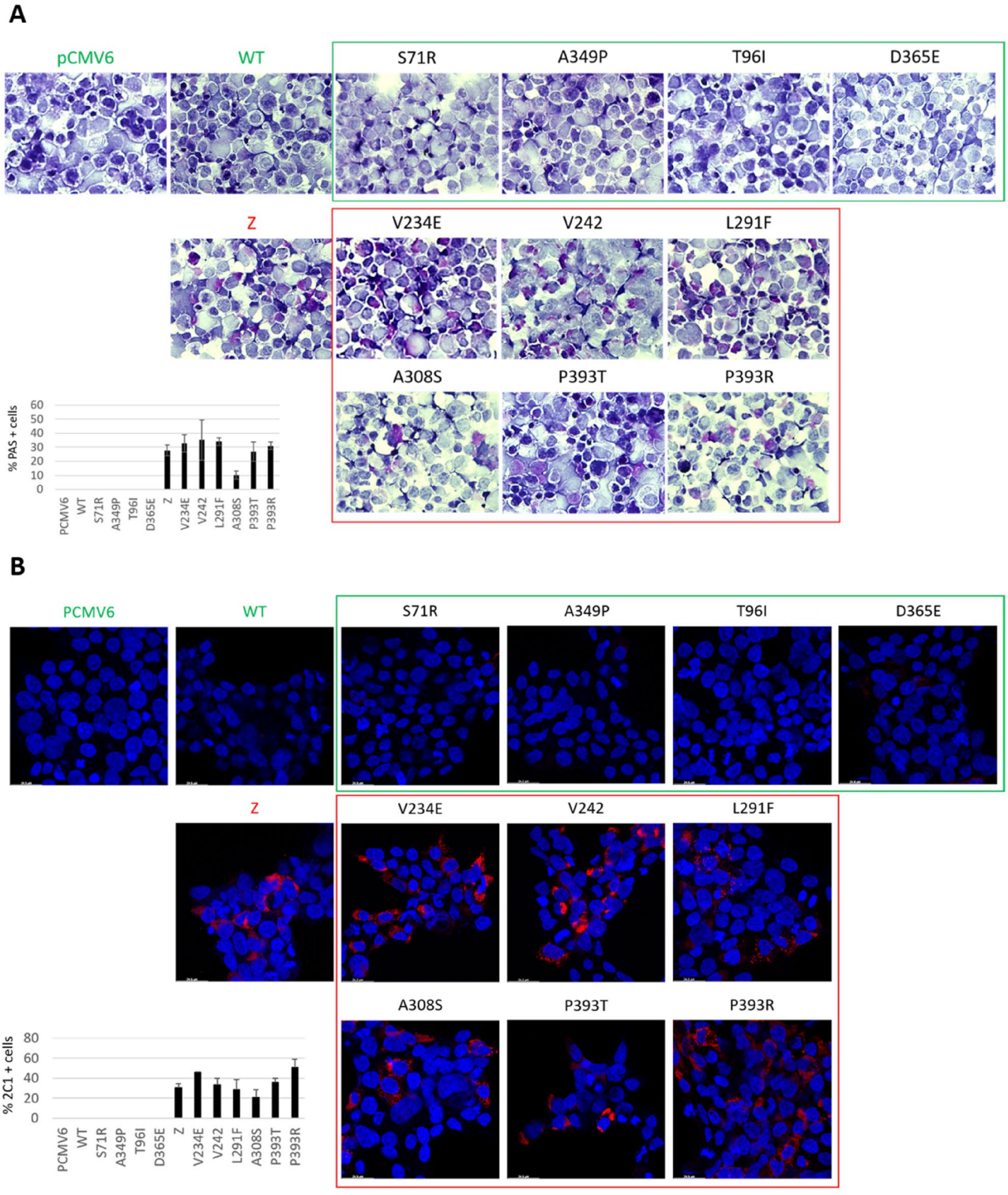


Fig. 2 Intracellular AAT polymers detected by (**A**) Periodic acid–Schiff (PAS) staining and (**B**) immunofluorescence staining using AAT polymer 2C1 antibody in HEK293T cells transfected with different AAT variants

p.Thr96Ile (Pi Getafe)

The proband was a 75-year-old female non-smoker with reduced serum AAT levels (59 mg/dL), under study due to a condition of altered intestinal rhythm. The respiratory function tests were normal (FVC: 83% predicted, FEV1: 91% predicted, FEV1/FVC: 76%, DLCO: 82% predicted, KCO: 86% predicted). Chest CT showed only small basal bronchiectasis predominantly in the left

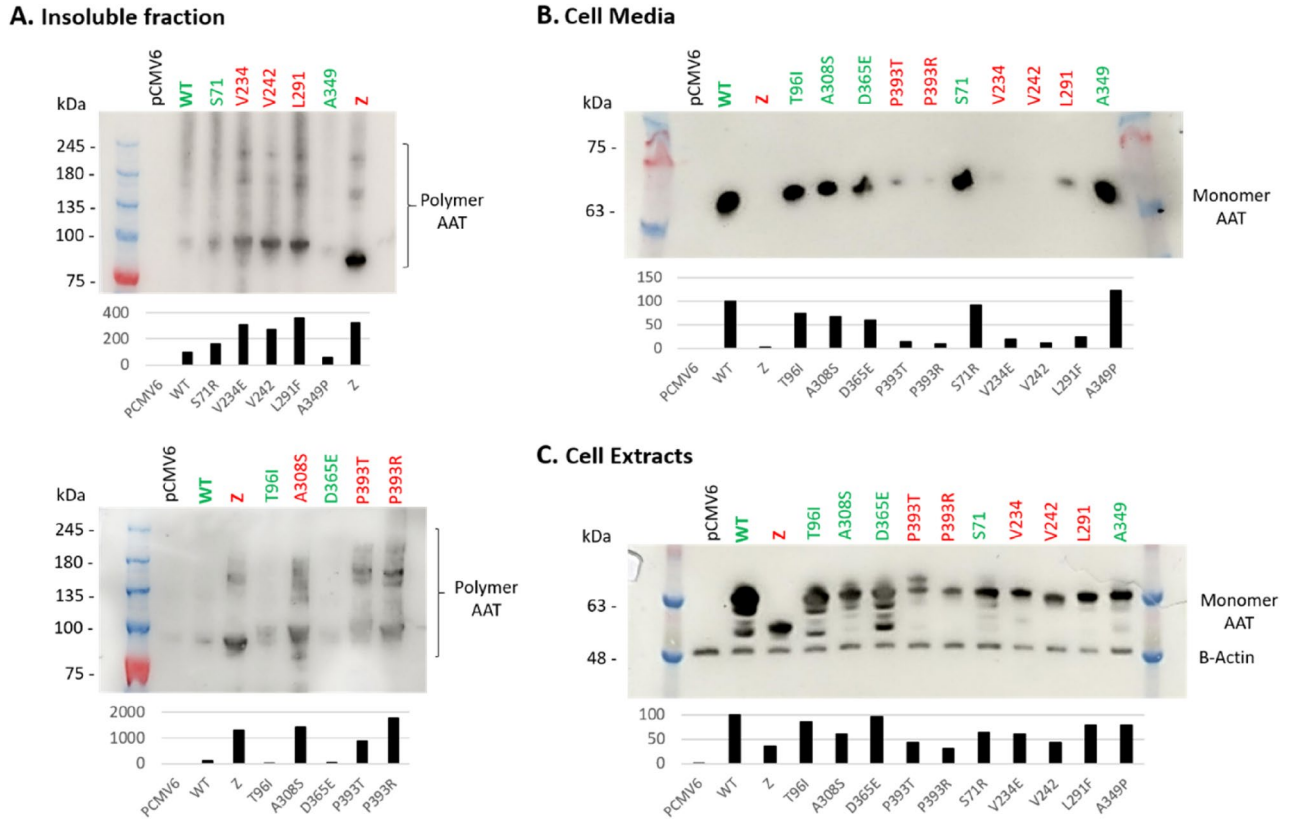


Fig. 3 Secretion and intracellular polymerization of AAT detected by Western blot analysis in HEK293T cells expressing different AAT variants. **A** Cell lysates were obtained and insoluble elements were analysed by non-denaturing PAGE. **B** Cell media of transfected cells was analysed by SDS-PAGE. **C** The soluble fraction of cell lysates was analysed by SDS-PAGE. Blots were detected with anti-AAT B9 (Santa Cruz Biotechnology) and anti–b-actin (Abcam) antibodies

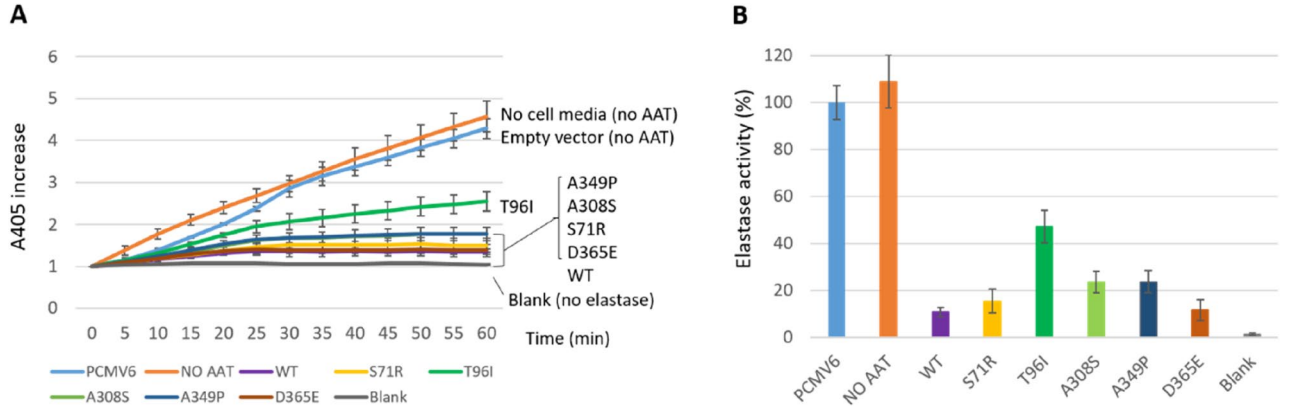


Fig. 4 Elastase inhibitory activity of AAT variants secreted into the cell media. **A** Culture media of cells expressing Ser71Arg, Thr96Ile, Ala308Ser, Ala349Pro and Asp365Glu variants was incubated with elastase and its substrate, and A405 increase was measured over time. Cells transfected with *SERPINA1* WT or an empty vector were used as controls. **B** Percentage of elastase activity of each variant compared with the empty vector (cell media without AAT). Percentages are averages (± SD) of three independent experiments

lower lobe. Liver function tests were normal. The A1AT genotyping test showed the presence of a heterozygous Z mutation. Given the discrepancy between serum AAT levels and this result, *SERPINA1* gene was completely sequenced, revealing Z mutation and c.287 C > T (p.Thr96Ile) variant. Familial co-segregation analysis demonstrated that both variants were in different alleles.

The p.Thr96Ile variant was predicted as likely pathogenic by three pathogenicity prediction algorithms (SIFT, Polyphen-2 and Alphamissense) and Varsome classified it as uncertain significance. The structural analysis

showed that p.Thr96Ile affects to a hydroxyl residue able to make a hydrogen bond with Lys334, which may be loss with an aliphatic Ile change. The substitution is at a huge distance (> 38 Å) to the key reactive center loop (RCL) and therefore might not be directly involved in polymerization (Fig. 1). The in vitro studies demonstrated that cells expressing variant p.Thr96Ile were negative for PAS staining (Fig. 2A) and for anti-AAT polymer 2C1 antibody (Fig. 2B), and did not show intracellular accumulation of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A).

When secretion efficiency of AAT was analysed by western blot in the culture media of transfected cells, the variant demonstrated efficient secretion into the medium (Fig. 3B). However, the elastase inhibition assay performed in the cell media of secreted variants demonstrated a significantly reduced inhibitory activity of p.Thr96Ile variant, producing an elastase inhibition of only 53% relative to the empty vector–expressing cells (Fig. 4). In conclusion, p.Thr96Ile variant showed no AAT polymerization, normal secretion but reduced anti-elastase activity when studied in vitro, so we have classified it as a likely pathogenic variant and consider it to represent a dysfunctional allele.

p.Val234Glu (Pi Viladecans)

This case was a 29-year-old female followed in the Hepatology Unit because of an alteration in liver enzymes (ALT: 73 IU/L, GGT: 75 IU/L). Both CT and Ultrasound showed signs of hepatic steatosis. The patient was non-diabetic, reported no regular alcohol intake and had a BMI of 28.5. She was a non-smoker with no respiratory symptoms and normal pulmonary function tests, but with reduced AAT serum levels (50 mg/dL). The A1AT genotyping test did not reveal any of the 14 mutations evaluated. Complete sequencing of the *SERPINA1* gene showed an allelic variant c.701T > A (p. Val234Glu).

The p.Val234Glu variant was predicted as likely pathogenic by three pathogenicity prediction algorithms (SIFT, Polyphen-2 and Alphamissense) and Varsome classified it as uncertain significance. The structure localization showed that p.Val234Glu introduced a charged group, able to make new saline bounds with the nitrogen atom of Asn214, which may eventually affect the RCL (Fig. 1). The in vitro studies demonstrated that cells transfected with p.Val234Glu variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and presented high levels of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A). In addition, the variant showed extremely low AAT secretion into the medium of transfected cells (Fig. 3B), despite being present inside the cell (Fig. 3C). In brief, p.Val234Glu variant showed AAT polymerization and impaired secretion when studied in vitro, so we have classified it as likely pathogenic, being a deficient allele.

It should be noted that p.Val234Glu variant has been previously reported [14], although no functional study was performed.

p.Val242_Pro243insLeu (Pi Cazorla)

This patient was a 74-year-old male, ex-smoker for 20 years with a cumulative tobacco use of 80 pack-years, and reduced AAT serum levels (58 mg/dL). Chest X-ray showed signs of emphysema and respiratory function tests revealed obstruction and functional restriction with diffusion impairment (FVC: 52% predicted, FEV1: 43% predicted, FEV1/FVC: 64%, DLCO: 51% predicted, TLC: 93% predicted, RV: 169% predicted). No alteration in liver enzymes was found. The patient underwent an A1AT genotyping test without observing any of the 14 mutations evaluated. Complete sequencing of the *SERPINA1* gene showed a heterozygous variant c.725_727dup (p. Val242_Pro243insLeu).

The p.Val242_Pro243insLeu variant was classified by Varsome as uncertain significance. The structural analysis showed that this insertion may directly alter the secondary structure of a beta-sheet. The flanking residues of this insertion are at contact distances of Thr392 (4.2 Å) and Lys290 (4.0 Å), respectively. Remarkably, Lys290 is also at charged bond distance (5.9 Å) with Asp341 and is apparently an important residue maintaining the structure of the protein surrounding the RCL. Despite the inserted position residue is oriented outwards without any measurable connection, it enlarges the RCL. This may potentially increase the relative flexibility between two independent beta sheets connected by this short region (Fig. 1). The functional studies demonstrated that cells expressing the p.Val242_Pro243insLeu variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and formed AAT polymers detected by western blot (Fig. 3A). In addition, the variant showed absent AAT secretion into the medium of transfected cells (Fig. 3B), in spite of being present inside the cell (Fig. 3C). In conclusion, p.Val242_Pro243insLeu variant showed AAT polymerization and impaired secretion when studied in vitro, so we have classified it as likely pathogenic, being a deficient allele.

This variant was previously mentioned by our group [15], although no functional study was performed.

p.Gln285Ter (Q0 Donosti)

The proband was a 48-year-old male, ex-smoker with 5 pack-years and low AAT serum levels (64 mg/dL). Chest x-ray and respiratory function tests were normal (FEV1: 97% predicted, FEV1/FVC: 80%, DLCO: 89% predicted, KCO: 102% predicted). Liver tests: GGT 110 IU/L, other liver enzymes were normal. The A1AT genotyping test

was performed without observing any of the 14 mutations evaluated. *SERPINA1* complete sequencing showed an allelic variant c.853 C > T (p.Gln285Ter).

The p.Gln285Ter is the result of a bp substitution in codon 285, causing a premature stop codon. This variant is likely to affect the mRNA processing or truncated protein stability and was deemed a null allele; therefore, we classified it as as pathogenic. This variant was previously mentioned by our group [15].

p.Leu291Phe (Pi São Mateus do Sul)

This patient was a 69-year-old male under study for dyspnoea and frequent exacerbations, smoker with 50 pack-years. Respiratory function tests showed an obstructive pattern (FEV1: 52% predicted, FEV1/FVC: 45%) and altered CO transfer (DLCO: 31.5% predicted, KCO: 41% predicted). Chest X-ray and thoracic CT showed predominantly apical emphysema. Blood tests revealed low levels of AAT (54 mg/dL). Liver function tests showed normal values. The patient underwent an A1AT genotyping test without observing any of the 14 mutations evaluated. Complete sequencing of the *SERPINA1* gene showed an allelic variant c.871 C > T (p. Leu291Phe).

The p.Leu291Phe variant was predicted as likely pathogenic by SIFT and Polyphen-2 but likely benign by Alphamissense. The structural analysis showed that the substitution of an aliphatic by a bulkier Phe residue might favor aromatic-aromatic interaction with Phe277 residue, for which all benzene carbons located at ≤ 5.2 Å (Fig. 1). The in vitro studies demonstrated that cells transfected with p.Leu291Phe variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and presented high levels of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A). In addition, the variant showed extremely low AAT secretion into the medium of transfected cells (Fig. 3B), in spite of being present inside the cell (Fig. 3C). In brief, p.Leu291Phe variant showed AAT polymerization and impaired secretion when studied in vitro, so we classified it as likely pathogenic and results in a deficient allele.

p.Ala308Ser (M1 Lyon)

The proband was a 72-year-old male, ex-smoker, with a family history of AATD and low serum levels of AAT (43 mg/dL). He was followed due to a chronic obstructive disease GOLD 3B BODE 4. CT scan showed signs of air trapping, basal bronchiectasis and elevation of left hemidiaphragm. Liver function tests were normal. Respiratory function tests showed FEV1: 41% predicted, FVC: 43% predicted, FEV1/FVC: 67%, DLCO: 49% predicted, KCO: 78% predicted, VA: 61% predicted. A1AT genotyping test showed the presence of a heterozygous S mutation. Complete sequencing of *SERPINA1* gene revealed S mutation and c.922G > T (p.Ala308Ser) variant. Familial co-segregation analysis demonstrated that both variants were in *trans*.

The p.Ala308Ser variant was predicted as deleterious by SIFT but benign by Polyphen, Alphamissense and Varsome tools. The structural mapping showed that the hydroxyl group added by this amino acid substitution may mediate hydrogen bonds with the Proline at 362 position in the RCL (Fig. 1). The functional studies demonstrated that cells transfected with p.Ala308Ser variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and presented high levels of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A). The variant showed a lower percentage of PAS- and 2C1-positive cells, compared to the rest of the positive variants, suggesting the generation of a less polymerogenic protein or polymers that are more unstable. Consequently, p.Ala308Ser variant was present in the culture media (Fig. 3B).

The elastase inhibition assay in the cell media of secreted variants showed that the p.Ala308Ser variant inhibited elastase activity by 77%, similar to the inhibitory activity of WT AAT and other secreted variants (Fig. 4). In conclusion, p.Ala308Ser variant showed AAT polymerization but secretion and normal anti-elastase activity when studied in vitro, so we have classified it as likely pathogenic.

It is noteworthy that p.Ala308Ser variant has been previously described [13, 16] and classified as likely benign and uncertain significance, respectively, although no functional study was performed. Since this variant polymerizes, we believe it may cause a risk and should be re-classified as mildly deficient.

p.Ala349Pro (Pi Almirante Tamandaré)

The patient was a 71-year-old female, former smoker, followed in the Pulmonology Unit for COPD, without alteration of liver function. A1AT genotyping test was performed when she reported low AAT serum levels, but none of the 14 mutations was detected. *SERPINA1* gene sequencing showed an allelic variant c.1045G > C (p.Ala349Pro).

The p.Ala349Pro variant was predicted as benign by three pathogenicity prediction algorithms (SIFT, Polyphen-2 and Alphamissense) and Varsome classified it as likely benign. The structural analysis showed that the side chain at this position is oriented to the solvent without any contact with other residues, and far away from the key RCL (Fig. 1), suggesting low structural impact of the mutation.

The in vitro studies demonstrated that cells expressing variant p.Ala349Pro were negative for PAS staining (Fig. 2A) and anti-AAT polymer 2C1 antibody (Fig. 2B), and showed absence of AAT polymers in the insoluble fraction of transfected cells when analysed by western

blot (Fig. 3A). In addition, transfected cells demonstrated efficient AAT secretion into the medium (Fig. 3B). Finally, the elastase inhibition assay in the cell media of secreted variants showed that the p.Ala349Pro variant inhibited elastase activity by 77%, similar to the inhibitory activity of WT AAT and other secreted variants (Fig. 4). To sum up, p.Ala349Pro variant showed no AAT polymerization, normal secretion and normal anti-elastase activity when studied in vitro; therefore, we have classified it as a likely benign variant and consider it to represent a normal AAT allele. AAT levels were requested again from the patient and were found to be normal (117 mg/dL).

p.Asp365Glu (Pi Istambul)

The proband was a 24-year-old male, non-smoker, who reported low levels of AAT (60 mg/dL). A1AT genotyping test was negative and *SERPINA1* complete sequencing showed c.1095 C > G (p.Asp365Glu) variant.

The p.Asp365Glu variant was predicted as likely pathogenic by SIFT and Alphamissense but benign by Polyphen, and Varsome classified it as uncertain significance. This was the only conservative, i.e. acidic by acidic side chains, substitution in our mutation dataset. The structural analysis showed the carboxyl group of this acidic residue is at only 4.5 Å of the positive charged NZ atom of Lys215, indicating they interact through a saline bond. However, the addition of an internal methyl group within the side chain due to the aspartic-to-glutamic acid substitution would not likely modify this bridge as interacting atoms become even closer, 3.1 Å (Fig. 1).

The functional studies demonstrated that cells expressing variant p.Asp365Glu were negative for PAS staining (Fig. 2A) and anti-AAT polymer 2C1 antibody (Fig. 2B) and showed absence of AAT polymers in the insoluble fraction of transfected cells when analysed by western blot (Fig. 3A). In addition, transfected cells demonstrated efficient AAT secretion into the medium (Fig. 3B). Finally, the elastase inhibition assay in the cell media of secreted variants showed that the p.Asp365Glu variant inhibited elastase activity by 89%, similar to the inhibitory activity of WT AAT and other secreted variants (Fig. 4). In conclusion, p.Asp365Glu variant showed no AAT polymerization, normal secretion and normal anti-elastase activity when studied in vitro; therefore, we have classified it as a likely benign variant and consider it to represent a normal AAT allele. AAT levels were requested again from the patient and were found to be normal (142 mg/dL).

p.Pro393Thr (Pi Complutense)

The patient was a 53-year-old woman, ex-smoker with 35 pack-years and low AAT serum levels (40 mg/dL). Liver function and respiratory function tests (FEV1: 121% predicted, FVC: 130% predicted, FEV1/FVC: 79%, DLCO: 93% predicted, KCO: 85% predicted) were within normal ranges, despite the fact that the patient had not undergone pulmonary transplantation. A1AT genotyping test showed a heterozygous M/Z genotype. Complete sequencing of *SERPINA1* gene detected an additional allelic variant c.1177 C > A (p.Pro393Thr) on different allele than Z, so that genotype was Z/p.Pro393Thr.

The p.Pro393Thr variant was predicted as likely pathogenic by SIFT, Polyphen and Varsome tools and ambiguous by Alphamissense. The structural mapping showed that the change involved the replacement of a Pro by a residue with a hydroxyl group, able to make new hydrogen bonds, probably with Asn70 or Lys411, in a position close to the RCL (Fig. 1).

The in vitro studies demonstrated that cells transfected with p.Pro393Thr variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and presented high levels of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A). In addition, the variant showed extremely low AAT secretion into the medium of transfected cells (Fig. 3B), in spite of being present inside the cell (Fig. 3C). In brief, p.Pro393Thr variant showed AAT polymerization and impaired secretion when studied in vitro, so we have classified it as a pathogenic variant and consider it to represent a deficient allele.

p.Pro393Arg (Pi Ponferrada)

The patient was a 60-year-old female, former smoker with 50 pack-years and reduced AAT serum levels (60 mg/dL). She was followed in the Pulmonology Unit (FVC: 107% predicted, FEV1: 108% predicted, FEV1/FVC: 64% predicted, DLCO: 54% predicted, KCO: 54% predicted). Not known liver disease. A1AT genotyping test showed none of the 14 mutations. *SERPINA1* complete sequencing detected a homozygous c.1178 C > G (p.Pro393Arg) variant.

The p.Pro393Arg variant was predicted as damaging by three pathogenicity prediction algorithms (SIFT, Polyphen-2 and Alphamissense) and Varsome classified it as likely pathogenic. The structural mapping showed that this variant involved the replacement of a Pro by a residue with a positively charged side chain, able to make new salt bonds among other ionic effects, in a position close to the RCL (Fig. 1).

The functional studies demonstrated that cells transfected with p.Pro393Arg variant were positive for PAS staining (Fig. 2A) and AAT polymer 2C1 antibody (Fig. 2B) and presented high levels of polymerogenic AAT in the insoluble fraction when analysed by western blot (Fig. 3A). In addition, the variant showed extremely low AAT secretion into the medium of transfected cells (Fig. 3B), in spite of being present inside the cell (Fig. 3C). In conclusion, p.Pro393Arg variant showed AAT

polymerization and impaired secretion when studied in vitro; therefore, we have classified it as a pathogenic variant and consider it to represent a deficient allele.

Discussion

Alpha-1 Antitrypsin Deficiency (AATD) is a genetic condition characterized by significant clinical variability and variable penetrance, posing a diagnostic challenge. The clinical variability of AATD is, in part, due to the significant genetic variability of *SERPINA1*, among other factors. The intrinsic flexibility of serpins makes them vulnerable to point mutations, which can lead to abnormal intra and intermolecular connections, affecting the folding, secretion and/or inhibitory function of the protein [17].

This study describes and characterizes thirteen novel rare *SERPINA1* variants associated with AATD, thereby expanding the spectrum of mutations linked to this condition. The identification and functional characterization of new genetic variants primarily strengthens the diagnostic process by improving the accuracy of molecular testing and deepening our understanding of the pathogenic mechanisms underlying the deficiency [14, 18, 19]. These findings may also assist in supporting patient stratification based on genetic profiles, offering complementary information that can be considered within the broader clinical assessment. Although these insights do not modify current recommendations for clinical management or treatment, they help inform prognostic evaluation and may contribute, in the long term, to more individualized follow-up strategies. Moreover, genetic diagnosis enables the identification of carriers and asymptomatic relatives before symptom onset, facilitating early implementation of preventive measures—such as smoking cessation, avoidance of respiratory irritants, and limiting exposure to alcohol or other hepatotoxic agents—which can contribute to reducing the risk of pulmonary and hepatic damage.

As next generation sequencing is more widely implemented in clinical practice, more variants are being detected. This necessitates additional research to understand the impact of the numerous genetic alterations of *SERPINA1* gene on AAT protein structure and function. Missense variants remain one of the most challenging types of genetic alterations to interpret, in many cases requiring a multifaceted approach for accurate evaluation. Currently, ClinVar, a database that aggregates and curates information about genetic variants and their clinical significance, reports a large number of *SERPINA1* exonic variants that are classified as having uncertain significance (65 variants) and many variants with conflicting classifications (21 variants). This highlights the need to further explore the effect of the numerous variants found in patients with additional tools other than the established pathogenicity predictors.

Recently, next-generation sequencing in combination with protein modeling and computational prediction tools have been used to identify and assess the pathogenicity of new *SERPINA1* rare variants. In some cases, some in silico tools showed high accuracy in predicting pathogenicity and were subsequently confirmed through cell model experiments [14, 20–22]. Molecular dynamics simulations have revealed that certain missense variants can significantly impact protein stability, structure, and function [23]. However, the performance of these predictive tools can vary depending on the specific type of mutation, with some tools showing lower sensitivity for certain variant types.

In this work, we have been able to test the accuracy of in silico pathogenicity predictors such as SIFT, Polyphen and AlphaMissense. Although in silico predictors are useful in general terms, our results indicated that they sometimes present inconsistent results and can generate false positives or negatives, so it is necessary to complement them with experimental and clinical data. This was the case of the p.Ala308Ser variant (M1 Lyon), which was classified as benign by Polyphen and Alphamissense and as likely benign in the literature [13], but the functional study showed some polymerization and retention in the cell, prompting us to classify it as a mild-deficient allele. Another example is the p.Asp365Glu variant (Pi Istanbul), classified as deleterious by SIFT and Alphamissense, but in vitro studies showed no polymerization, normal secretion and normal anti-elastase activity.

When predicting the impact of genetic variants, caution is warranted, since different variants affecting the same codon could have different effects, depending on the amino acid substitution. On the other hand, there might be essential residues in the protein, where any change is likely to be pathogenic, as in the case of residue Pro393. In our study, we found two variants affecting this residue, both being pathogenic: p.Pro393Thr (Pi Complutense) and p.Pro393Arg (Pi Ponferrada). Other Pro393 substitutions have been described in the literature and classified as pathogenic: p.Pro393Leu (MHerleen) [24], p.Pro393Ser (MWurzburg) [24] and p.Pro393Arg (Wdecatur) [25].

The structural mapping of the variants identified in this study has revealed that polymerization-associated mutations might modify the protein dynamics mostly in regard to new bonds in the apical region, near the RCL, which eventually increases the affinity constant for self-interaction. Conversely, benign variants were usually far from the RCL, outwardly oriented or involved conservative amino acid substitutions. The mechanisms of serpin polymerization and the position of mutations relative to the RCL has been previously studied, especially for the

common severe Z mutant [26–28]. Knowing whether a genetic variant is capable of producing AAT polymerization is key, not only to anticipate possible clinical consequences (e.g. liver damage), but also to identify therapeutic solutions aimed at inhibiting polymerization.

The only variant in our study affecting AAT anti-elastase activity (p.Thr96Ile) was at a huge distance from the RCL, in opposition to the polymerization-driven AATD mutations identified. This variant is located in the same region as the previously identified dysfunctional variant Pi Valencia (Lys328Glu) [11], both at the end of the β-sheet s5A. This region might be critical for AAT inhibitory activity, in addition to the RCL, in which inactivating substitutions have already been described [29].

Finally, it is necessary to consider some limitations of this study. The identification of new mutations was made through the Progenika Diagnostic Network, which includes samples from six countries [7]. The degree of AATD screening in these countries is variable and likely suboptimal, meaning the observed mutations are evaluated in patients with suspected AATD and probably underestimated the actual frequency in the general population. Another note of caution is warranted in cases of liver disease with an unknown cause. Most physicians participating in this network were pulmonologists or general practitioners, which may lead to an underrepresentation of such liver disease cases. Including liver disease specialists in the evaluation of these patients could enhance the detection of AATD cases in this clinical context.

Another limitation of our work is the absence of longitudinal patient follow-up and the lack of data on the clinical or epidemiological penetrance of the variants studied. These variants are very rare, and no population-based studies are available to determine their long-term clinical impact. Consequently, our conclusions are based exclusively on in vitro and in silico functional analyses, which, while informative, cannot substitute for clinical evidence.

In conclusion, we have described and characterized thirteen rare mutations associated with AATD (eight not previously described), expanding the spectrum of genetic variability linked to AATD. Functional assays are essential for establishing the pathogenicity of genetic variants, providing direct biological evidence that complements the predictions made by computational methods, and clinical data of the patients.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
NM performed experiments, analysed data, interpreted results and drafted the manuscript; LO performed experiments, analysed data and interpreted results; ED collected clinical data and interpreted results; SGM performed experiments and analysed data; AJMG performed protein structure analysis and interpreted the results; VAP, MJC, FC, CSS, MC, MSF, JOS, JALLQ, NRL, FJMR, JLRH, LSP and EA included cases and reviewed clinical data; JA, CBB and GGM interpreted results; JLLC and BMD interpreted results, drafted the manuscript, and revised the manuscript critically; all authors revised and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
This study reports the results of a diagnostic procedure that is approved for use in all the participating countries and, therefore, constitutes standard clinical practice. Consequently, since it is not an experimental procedure, approval by an ethics committee was not considered necessary. All patients included signed a written informed consent form.

Consent for publication
Signed informed consent was obtained from patients.

Competing interests
LO is full-time employee of Progenika, ED is full-time employee of Grifols.

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