UNIVERSIDAD DE MURCIA
FACULTAD DE MEDICINA

"New Mechanisms Involved in Antithrombin Deficiency: Relevance of N-glycosylation"

"Nuevos Mecanismos Implicados en la Deficiencia de Antitrombina: Relevancia de la N-glicosilación"

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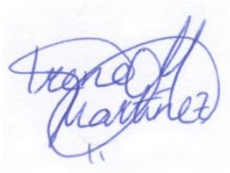
CERTIFICAN:

Que la tesis Doctoral titulada "**Nuevos mecanismos implicados en la deficiencia de antitrombina: Relevancia de la N-glicosilación**", realizada por Dª. **Sonia Águila Martínez** bajo su inmediata dirección y supervisión, y que se presenta para la obtención del grado de Doctor Internacional por la Universidad de Murcia.

En Murcia, a 12 de Septiembre de 2014



Fdo: D. Javier Corral de la Calle

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En Murcia, a 12 de Septiembre de 2014

Fdo: Sonia Águila Martíne

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“Actúa de la manera en la que te gustaría ser y pronto serás de la manera en la que actúas”

Leonard Cohen

“Ciencia es todo aquello sobre lo cual siempre cabe discusión”

José Ortega y Gasset

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Abbreviations:

ACN: acetonitrile

Ag: antigen levels

Anti-Xa/IIa or anti-FXa/FIIa activity: antithrombin activity towards factor Xa or factor IIa, in presence of heparin, unless otherwise indicated.

AT: antithrombin

AT*: cleaved antithrombin

BCA: bicinchoninic acid

ΔCp: denaturation delta capacity change

CD-CHO: chemically-defined medium optimized for the growth of chinese hamster ovary

CHO: chinese hamster ovary cell line.

COS: monkey fibroblast-like cell line, obtained by immortalizing CV-1 (monkey kidney cells) cells with a version of the SV40 virus that coding for wild-type T antigen.

cDNA: complementary deoxyribonucleic acid

DNA: deoxyribonucleic acid

DMEN-glutaMAX: Dulbecco's modified Eagle medium with glutamine

DSC: differential scanning calorimetry

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

ER: endoplasmic reticulum

FPLC: fast protein liquid chromatography

FXa: active factor X

FIIa: active factor IIa

GAGs: glycosaminoglycans

Glc: glucose

GlcNAc: N-acetylglucosamine

HBS: heparin binding site

HEK-EBNA: human embryonic kidney cell line expressing the Epstein Barr nuclear antigen 1

HPLC: high-performance liquid chromatography

HNE: human neutrophil elastase

I: ionic strength

Ig: immunoglobulin

Kapp: apparent rate constant

K_D: dissociation constan

MALDI-TOF-MS: (MALDI) matrix-assisted laser desorption/ionization, (TOF-MS) time-of-flight mass spectrometer

Man: mannose

NGF or PNGase: N-glycosidase F

NP-40: 4-nonylphenyl poly(ethylene glycol)

OST: oligosaccharyl transferase

PBS: phosphate buffered saline

pI: isoelectric point

RCL: reactive center loop

RNA: ribonucleic acid

SD: Standart desviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SI: stoichiometry

s: stand (i.e. s4C: strand 4C)

TFA: trifluoroacetic acid

Tm: Melting temperature

UFH or H: unfractionated heparin

V1/2: variant 1 or 2

WT: wild-type

INTRODUCTION

1. Haemostatic system

The haemostatic system is the result of 430 millions of years of evolution to avoid the deleterious consequences of vascular damage in organisms with a closed and highly pressurized circulatory system. Platelets and the procoagulant cascade trigger a response by exposition of tissue factor and fibrinogen after vascular damage, which results in series of amplification and activation of multiple serine proteases whose final actor is thrombin, the enzyme that forms the fibrin clot (Figure 1) (Stassen *et al.*, 2004).

Many of the procoagulant proteases are subjected to control by serpins (SERin Protease INhibitors) and their local cofactors. There are four anticoagulant serpins: Protein C Inhibitor (SERPINA5), Protein Z-dependent protease inhibitor (ZPI, SERPINA10), heparin cofactor II (SERPIND1), and antithrombin (SERPINC1) (Figure 1).

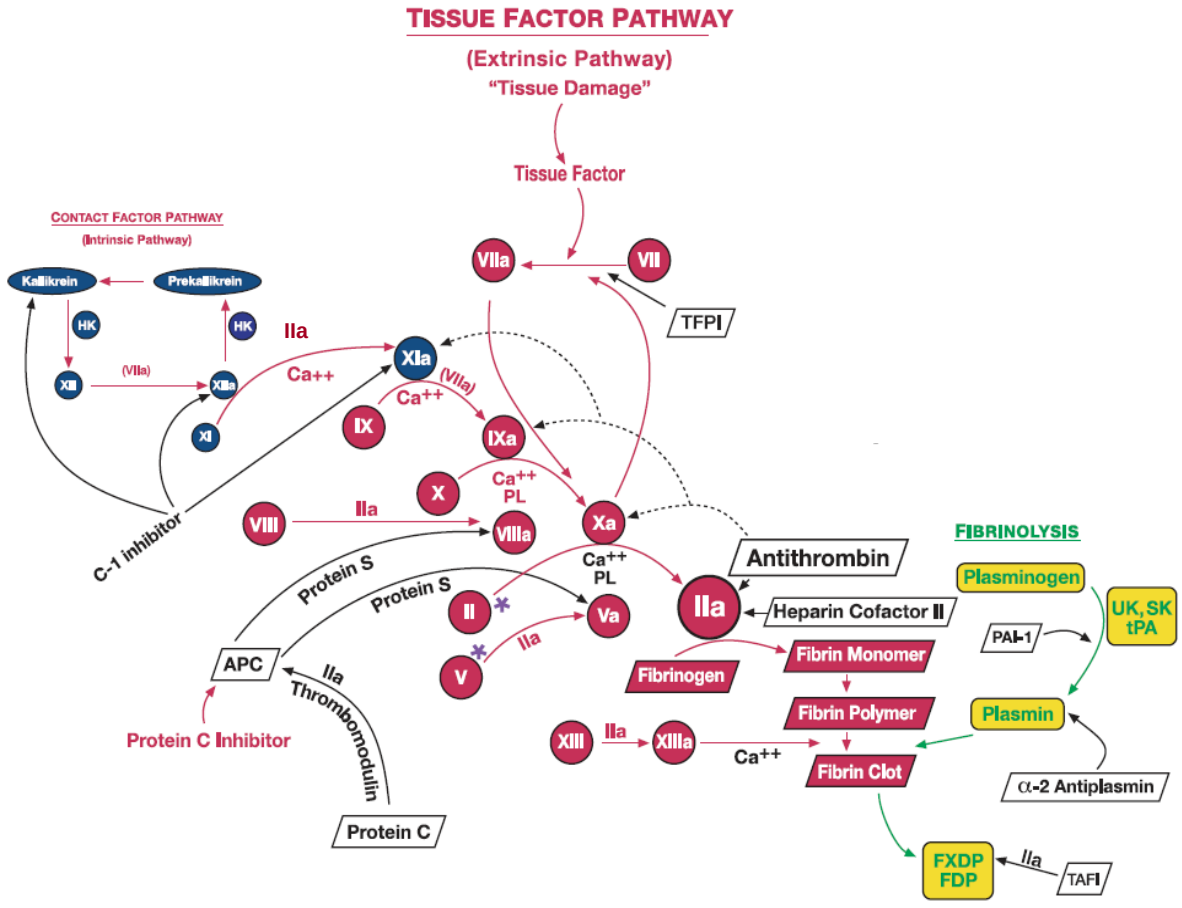


Figure 1. Blood clotting cascade.

2. Antithrombin

In 1965, O. Egeberg described the first thrombophilic defect, when reported the association of antithrombin deficiency with the incidence of venous thrombosis in a Norwegian family (Abildgaard, 2001; Egeberg, 1965). The strong risk of thrombosis associated with antithrombin deficiency, together with the study of the structural and functional features of this inhibitor gave strong evidence of the key role of this anticoagulant in the haemostatic system. Antithrombin has a relatively broad range of target proteases. Thus, it inhibits practically all procoagulant proteases and particularly the last two crucial proteases of the coagulation cascade: thrombin (FIIa) and factor Xa (FXa) (Figure 1).

2.1 Structural and functional features of antithrombin

Antithrombin is synthesized in the liver and secreted to the circulation, where it can reach levels about 150 μg/ml. The molecule has a plasma half-life of approximately 3 days. Antithrombin is a single-chain ~58 kDa molecule with 432 amino acids that contains four potential N-glycosylation sites (N96, N135, N155, N192) and three disulphide bonds (Figure 2).

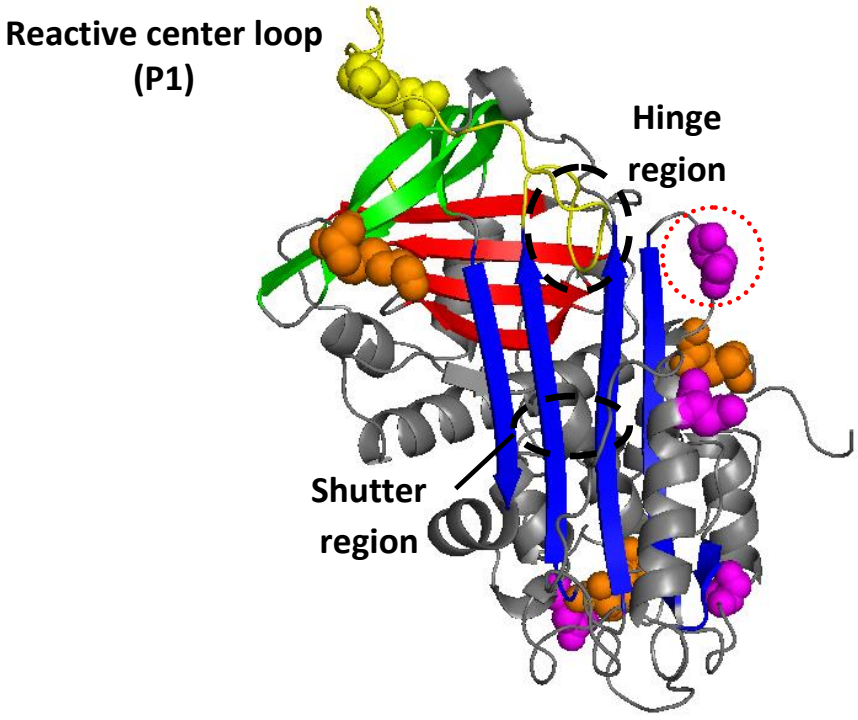


Figure 2. Ribbon diagram of native antithrombin (PDB code: 1T1F). Sheets A,B and C are displayed in blue, red and green respectively. The reactive center loop is in yellow, with P1 residue (R393) depicted as a yellow sphere. Cysteine residues involved in disulphide bonds are depicted as orange spheres. Asparagine residues involved in N-glycosylation are depicted in magenta spheres, with N135, which is not glycosylated in the β-isoform marked in red circle. The structure was rendered in Pymol (www.pymol.com).

Antithrombin, as a member of the serpin super-family, shares a conserved structure with three β-sheets (A–C) and nine α-helices (A–I). Antithrombin is characterized by its considerable flexibility, particularly that concerning the mobility of the reactive center loop (RCL), involved in the interaction with the target proteases, and the central A β-sheet (Irving *et al.*, 2000; Whisstock *et al.*, 1998)(Figure 2).

2.1.1 Inhibitory mechanism of serpins

The mechanism of inhibition, like for all members of the serpin superfamily, is extraordinary and highly efficient. The RCL of serpins acts as a protease-specific bait. The RCL cleavage of serpins by target proteases results in their inhibition by an efficient suicide mechanism that has been compared with a "mouse-trap" (Figure 3). Once the protease is bound, its catalytic serine attacks the reactive P1 (Arg in antithrombin), resulting in the formation of an acyl-intermediate complex in which the RCL has been cleaved but remains covalently linked to the catalytic serine. This cleavage triggers the suicide mechanism by freeing the metastable serpin to undergo a massive conformational change in which the cleaved RCL inserts into the center of the A sheet. This conformational change provokes the dragging of the protease to the opposite end of the serpin and it remains inactivated (Figure 3) (Dementiev *et al.*, 2013; Huntington *et al.*, 2000; Olson *et al.*, 2010; Stratikos *et al.*, 1999).

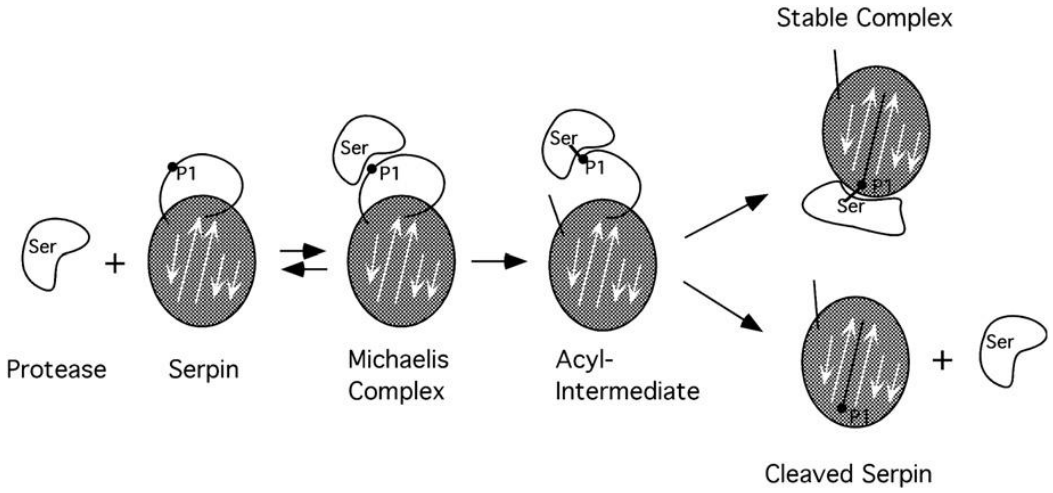


Figure 3. Branched pathway suicide substrate mechanism by which serpins inhibit serine and cysteine proteases. The serpin is gray-shaded; the protease is white drawing and the strands of β-sheet A show as arrows. *Olson ST, Richard B, Izaguirre G, Schedin-Weiss S, Gettins PG. Molecular mechanism of antithrombin-heparin regulation of blood clotting proteinase. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitor. Biochemie 2010.*

This mechanism is possible because serpins do not fold into their most thermodynamically favorable conformation. The serpin's native state is metastable, a kinetically trapped folding intermediate that upon RCL cleavage by proteases releases the energy for a significant

conformational transition, resulting in the more stable cleaved structure (Figure 4) (Cho *et al.*, 2005; Jin *et al.*, 1997; Langdown *et al.*, 2009). Moreover, antithrombin can spontaneously transform in a latent form, in which the RCL is inserted in the central A-sheet, but it is not cleaved (Mushunje *et al.*, 2004). Latent, cleaved and polymer conformations (see explanation afterwards) are detected electrophoretically in native PAGE in presence of 6M Urea, as they do not misfold (Mushunje *et al.*, 2004). Moreover, cleaved antithrombin shows a sift in its mobility in SDS-PAGE under non-reducing condition.

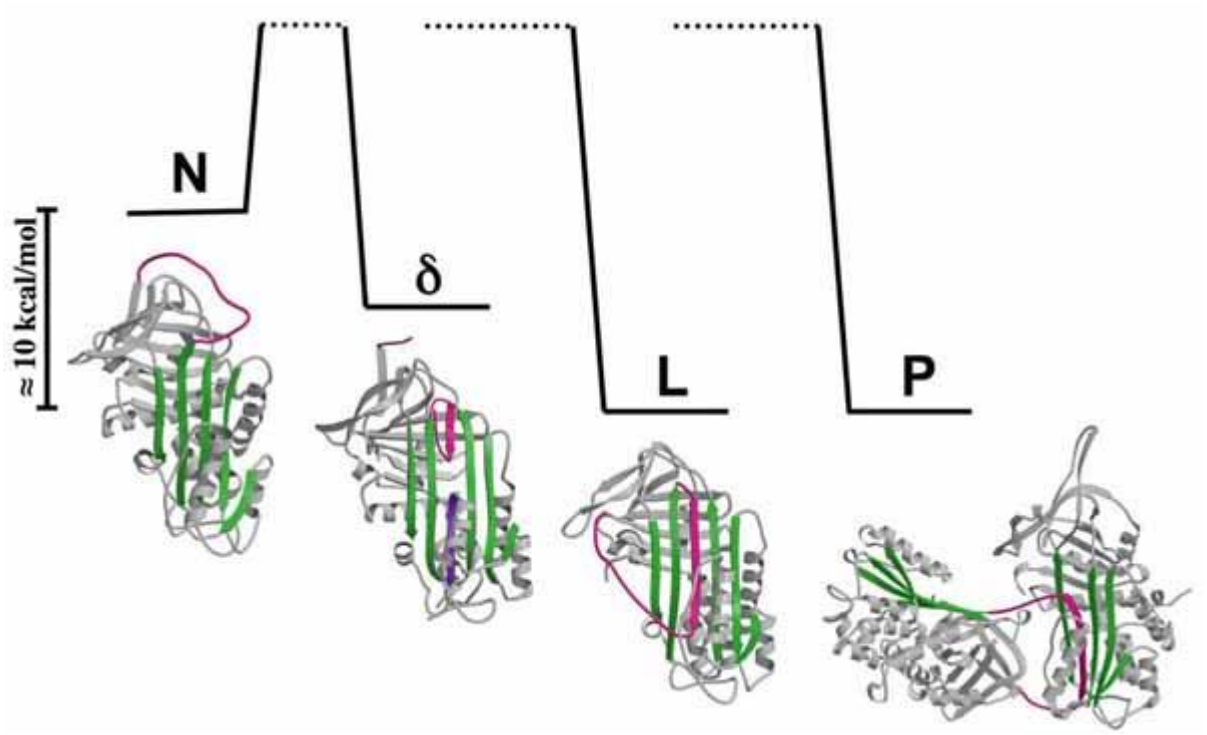


Figure 4. Energy diagrams illustrating alternative serpin conformations. The native state (N) represents the least stable conformation, followed by the intermediately stable δ conformation (α1-antichymotrypsin). The difference in stability between the latent (L) and polymeric (P) states is unknown, and they are presented here as being equally stable. *Devlin GL, Bottomley SP. A protein family under 'stress': serpin stability, folding and misfolding. Front Biosci. 2005*

Additionally, antithrombin presents also some attractive and repulsive exosites that exist outside the RCL. These exosites have differential accessibility in native and activated states of the serpin and let the regulation of proteases binding (Gettins *et al.*, 2009; Izaguirre *et al.*, 2009).

In contrast to other serpins, the RCL of native antithrombin remains partially inserted into the top of the central A β-sheet, showing a native stressed conformation (Carrell *et al.*, 1985). The region inserted is called hinge region (P9-P14) (Figure 2).

2.1.2 Regulation of antithrombin reactivity by glycosaminoglycans

In order to allow a right function of the haemostatic system, antithrombin is controlled by glycosaminoglycans (GAGs). The heparan sulfates type GAGs are located on the surface of blood vessel endothelial cells (luminal and subluminal) and they can activate circulating antithrombin (de Agostini *et al.*, 1990). As indicated before, the RCL is partially inserted into the central A-sheet in circulating antithrombin, and joined to the core of the molecule by a salt bridge between P1 (R393) and E237. This conformation explains the low anticoagulant activity of native antithrombin. Actually, equilibrium between the native and a partially activated conformation has been suggested based on the results observed when the R393-E237 salt bridge is broken (Johnson *et al.*, 2006a). However, under normal conditions the native form is predominant (Johnson *et al.*, 2006a). The binding of the essential pentasaccharide to the heparin binding site (HBS) of antithrombin, which involves the helix D, helix A and N-terminal, induces a complex set of conformational changes in the molecule that lead to the complete exposition of the RCL and exosites (Figure 5A). These changes accelerate the interaction of antithrombin with FXa by 270-fold (Olson *et al.*, 1992). However, antithrombin requieres the presence of full-length heparin for the full accelerated inhibition of its major target, thrombin (Olson *et al.*, 1992). This bridging mechanism accelerates the interaction with this serine proteases by up to 17,000-fold. The longer chain heparin acts as a bridge between protease and inhibitor by a combination of the pentasaccharide-induced conformational change of antithrombin and the simultaneous binding of the serine protease to the same chain of heparin forming a ternary complex (Figure 5B) (Olson *et al.*, 1992). The requirement of the binding of GAGs to the HBS of antithrombin to activate it allows a relative delay of the anticoagulant activity. Moreover, it also restricts the anticoagulant activity to the areas of vascular damage, and certainly contributes to maintain an equilibrated haemostatic system with procoagulant and anticoagulant capacity (Pike *et al.*, 2005). Finally, heparin is used as anticoagulant therapy in clinical settings due to the control on antithrombin activity.

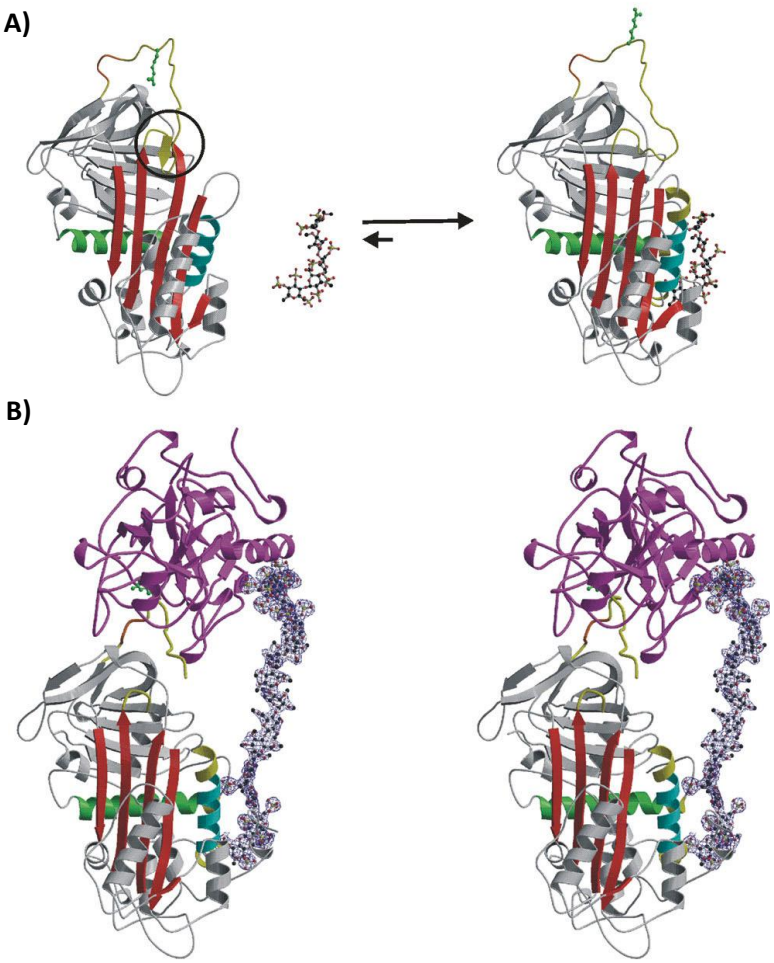


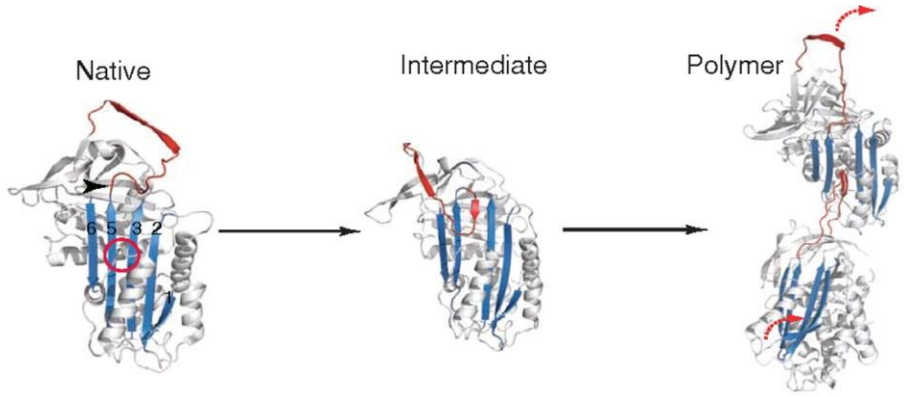
Figure 5. Heparin catalysis of thrombin inhibition by antithrombin. (**A**) The binding of the heparin pentasaccharide to antithrombin induces a global conformational change involving the expulsion of the hinge region (circled) of the reactive center loop (RCL, yellow) from the central A-sheet (red), extension (yellow) of the A and D-helixs (green and cyan, respectively) and P1 Arg (green stick). (**B**) Stereo representation of the crystal structure of the ternary complex between antithrombin (colored as above), thrombin (magenta) and heparin (balland-stick). *Li W, Johnson D, Esmon CT & Huntington JA. Structure of the antithrombin–thrombin–heparin ternary complex reveals the antithrombotic mechanism of heparin. Nature structural & molecular biology 2004.*

2.2 Conformational alterations

After formation of a Michaelis complex and the cleavage of P1-P1′ bond, the serpin undergoes a dramatic transition from a 'stressed to relaxed' (S → R) conformation. The complex and precise mechanism of inhibition makes serpins vulnerable to even minor modifications affecting their conformation. (Dementiev *et al.*, 2013; Huntington *et al.*, 2000). Missense mutations may cause aberrant conformational transitions, mainly resulting in a unique hyperstable ordered protein-protein linkage (Raja *et al.*, 2003). The polymerization of serpins is associated with a group of diseases called serpinopathies (Gooptu *et al.*, 2009) Homologous mutations in this super-family of proteins have similar consequences that result

in degenerative neuropathies, emphysema, angioedema, and other disorders (Carrell *et al.*, 1997). The archetypal and most studied serpinopathy is α_1-antitrypsin deficiency, in which the affected individuals can develop neonatal hepatitis and cirrhosis and early onset emphysema (Lomas *et al.*, 1992). The mechanism of *in vivo* serpin polymerization is still under discussion. Thereby, there are currently three main models (Figure 6): the first 'loop-sheet' model (Figure 6A) (Dunstone *et al.*, 2000; Lomas *et al.*, 1992; Sivasothy *et al.*, 2000), and two recently described models that are based on crystal structures. One model suggests that in these polymers, the RCL-strand 5A β-hairpin domain swaps among neighbor monomers (Figure 6B) (Yamasaki *et al.*, 2008). The other crystal structure that has been characterized corresponds to a circular trimer of α_1-antitrypsin formed in the presence of an engineered disulfide bond with a triple strand 1C–5B–6B domain swap among monomers (Figure 6C) (Yamasaki *et al.*, 2011). Antithrombin may form particular polymers, since it might form self-terminating disulfide linked dimers, which are not able to continue the process of polymerization (Figure 6D) (Corral *et al.*, 2004; Yamasaki *et al.*, 2008).

A)



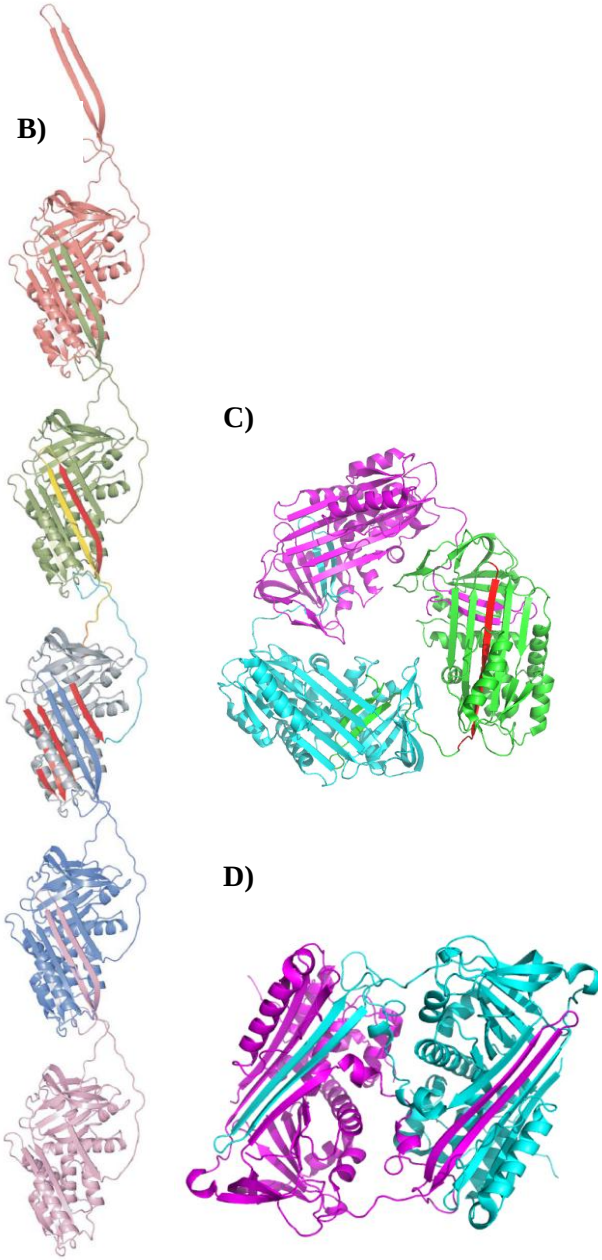


Figure 6. Models of serpin polymerization: **A)** The classical pathway of polymerization of α1-antitrypsin. "Loop-sheet" mode. *Ekeowa UI, Freeke J, Miranda E, Gooptu B, Bush MF, Pérez J, Teckman J, Robinson CV, Lomas DA. Defining the mechanism of polymerization in serpinopathies. Proc Natl Acad Sci U S A. 2010.* **B)** Model of pentamer or serpin polymer**.** *Yamasaki M, Li W, Johnson DJ, Huntington JA. Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization. Nature 2008.* **C)** Structure of an antitrypsin trimer formed via domain swapping of the C-terminal region (PDB code: 3T1P). *Yamasaki M, Sendall TJ, Pearce MC, Whisstock JC, Huntington JA. Molecular basis of α1-antitrypsin deficiency revealed by the structure of a domain-swapped trimer. EMBO Rep. 2011* **D)** Structure of a domain swapped antithrombin dimers reveals one mechanism of polymerization (PDB code: 2ZNH). *Yamasaki M, Li W, Johnson DJ, Huntington JA. Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization. Nature 2008.*

Other mutations, like those responsible for the variants Wibble and Wobble, did not impair a relatively correct folding and secretion of monomers, but they are conformational sensitive, transforming to the latent conformation (Beauchamp *et al.*, 1998). However, the wild-type antithrombin is able to spontaneously undergo a slow hyperstable transition by the insertion of the RCL that renders a latent or non-inhibitory antithrombin (Mushunje *et al.*, 2004). Up to 5% of circulating antithrombin has latent conformation.

2.3 Congenital deficiency of antithrombin

The critical role that antithrombin plays in controlling coagulation is reflected in the high risk of thrombosis (OR: 10-20) associated with heterozygous antithrombin deficiency states (Lane

et al., 1997). The relevance of antithrombin has been further illustrated by the lethal consequences of its complete deficiency during embryogenesis (Ishiguro *et al.*, 2000).

The high risk of a first thrombotic event and recurrence observed in carriers of congenital antithrombin deficiency together with the low incidence of bleeding when treated with oral anticoagulant therapy encourages to prolong their anticoagulant treatment. Moreover, the prophylaxis may benefit asymptomatic carriers under risk situations (Lijfering *et al.*, 2009; Mahmoodi *et al.*, 2010).

From a molecular point of view, a high mutation rate (>70%) in *SERPINC1*, the gene encoding antithrombin, has been described in patients with antithrombin deficiency (Luxembourg *et al.*, 2011). Most of these gene defects are heterozygous point mutations, although large gene deletions have been also identified (22-24). Only few cases with homozygous mutation have been described, which are associated with a high risk of thrombosis (Martinez-Martinez *et al.*, 2012c). Finally, only few reports have described compound heterozygosis (Emmerich *et al.*, 1994b; Perry *et al.*, 1995).

Antithrombin deficiencies are classified as **type I,** when there is not detected mutant antithrombin in plasma, resulting in 50% of antithrombin levels in circulation. This type of deficiency is caused by non sense, frameshift, or splicing mutations, as well as large deletions in *SERPINC1*. Some of them have been also explained by RNA instability. Interestingly, amino acid changes at crucial structural regions, particularly those affecting mobile regions (mainly the RCL or the shutter region involved in the opening of the main β-sheet), produce oligomer formation and intracellular retention, i.e. the cause of these antithrombin deficiencies is a conformational defect (Corral *et al.*, 2004; Corral *et al.*, 2005). Thus, some cases of thrombosis may be included within the group of conformational diseases (Corral *et al.*, 2005). Type I deficiency associates with strong risk of venous thrombosis.

Type II deficiencies are usually the consequence of missense single amino acid changes that result in a variant which is synthesized and secreted into the plasma with normal or reduced rate in comparison to wild type antithrombin, but the mutant molecule has an impaired or null anticoagulant activity. However, large insertions have also been described to cause type II deficiency (Martinez-Martinez *et al.*, 2012a). According to the mutation-induced effect, three varieties of type II deficiencies have been defined (Figure 7).

1) Type IIa: Reactive site defect. Mutations normally localized at the RCL impair the reactivity of the molecule, resulting in a loss of inhibitory activity in the absence or presence of heparin (Figure 7). These mutations usually associate with severe clinical phenotype.

Indeed, some of them, particularly those affecting the reactive P1 residue, lead to a complete loss-of-function. But, P1 mutations may also cause a gain of function reducing the function of wild type molecules in heterozygous subjcets by two mechanisms: by increasing the heparin affinity of the non-inhibitory variant and by infectivity. These functional effects explain why carriers of these mutations have early, recurrent and sometimes both venous and arterial thrombosis (Martinez-Martinez *et al.*, 2012b; Raja *et al.*, 2003).

2) Type IIb: Heparin binding defect. The mutation, usually located at the HBS, causes a low heparin affinity. Therefore, the mutant has normal progressive inhibitory activity in the absence of heparin, but it is not fully activated by heparin, with the consequent reduction of its anticoagulant capacity (Figure 8). Type IIb deficiencies have a mild clinical phenotype, thereby most homozygous cases described worldwide are patients with mutations affecting the HBS (Martinez-Martinez *et al.*, 2012c).

3) Type IIc: Pleiotropic defect. Mutations mainly located at the C-sheet that have multiple or pleiotropic effects on the heparin activation and the inhibitory activity, but the underlying mechanisms are not known. Lane and coworkers suggested that the altered heparin binding properties of s1C variants may reflect the conformational linkage between the reactive site and heparin binding regions of the molecule (Lane *et al.*, 1992).There is a considerable clinical variability among carriers of these mutations (Emmerich *et al.*, 1994a; Martinez-Martinez *et al.*, 2010; Olds *et al.*, 1992; Picard *et al.*, 2000).

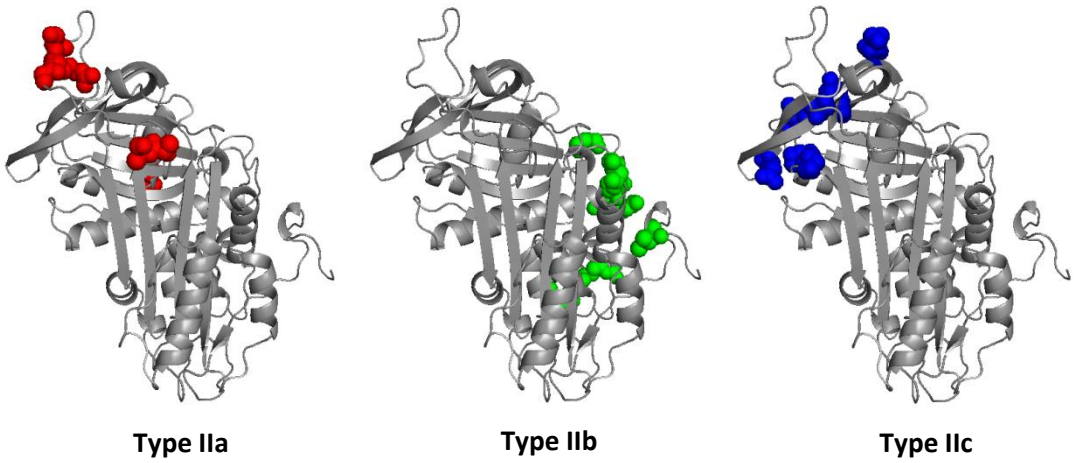


Figure 7. Localization of different mutations, which cause type II antithrombin deficiencies: IIa, IIb & IIc (green, magenta, blue spheres, respectively) in native antithrombin structure (PDB code: 1T1F). All structures were rendered in Pymol (www.pymol.com). The data were obtained in the web Imperial College London (www1.imperial.ac.uk.)

2.4 Antithrombin functions beyond hemostasis

Antithrombin is a serpin that has multiple biologically important properties beyond hemostasis (Roemisch *et al.*, 2002). Its potent anticoagulant function has been well demonstrated. Moreover, antithrombin also has remarkable anti-inflammatory properties. Some of these anti-inflammatory properties are secondary to its anticoagulant functions, as FXa and thrombin, targets of antithrombin, also contribute to inflammation; for instance, by the release of pro-inflammatory mediators. However, antithrombin has such anti-inflammatory properties by direct interactions with cellular receptor through HBS that trigger intracellular signaling, such as the release of prostacyclin *in vitro* and *in vivo* (Bae *et al.*, 2009; Dunzendorfer *et al.*, 2001; Harada *et al.*, 1999; Okajima *et al.*, 1998; Yang *et al.*, 2014). Binding of antithrombin to syndecan-4 leads to the interference with the intracellular signaling induced by mediators like lipopolysaccharides and, thereby, to a down-modulation of the inflammatory response (Kaneider *et al.*, 2003; Kaneider *et al.*, 2002; Oelschlager *et al.*, 2002). Indeed, the therapeutic use of antithrombin might be effective in patients with inflammatory disorders, including sepsis and critically ill patients, although using large supra-physiological doses without the concurrent use of any form of heparin (Levi *et al.*, 2008; Tagami *et al.*, 2014; Wiedermann, 2006). Moreover, our group has demonstrated an anti-apoptotic role for antithrombin that contributes to the protection of the liver to different insults (Guerrero *et al.*, 2012). Additional evidences suggest other functions associated with the conformational change of antithrombin. Thus, latent and cleaved antithrombin have strong anti-angiogenic properties as they can induce apoptosis of endothelial cells by disrupting cell-matrix interactions (O'Reilly, 2007; Richard *et al.*, 2008; Schedin-Weiss *et al.*, 2008).

3. Glycosylation

Proteins might have different post-translational modifications: acetylation, phosphorylation, lipidation, ubiquitination, proteolysis, or glycosylation. Glycosylation is the principal post-translational modification for most of proteins. The processing of carbohydrates bound to the proteins occurs between the lumen of the endoplasmic reticulum and the Golgi cisternae. Thus, the presence of certain carbohydrate residues on proteins provides useful markers for following their movement from the endoplasmic reticulum through the Golgi. There are two types of glycosylation: O- and N-glycosylation (Preston *et al.*, 2013; Yan *et al.*, 2005). The O-linked oligosaccharides are coupled to the hydroxyl group of serine or threonine *via* N-acetylgalactosamine or (in collagens) to the hydroxyl group of hydroxylysine *via* galactose. However, the O-glycosylation has not a clear consensus sequence. It is only known that the

oligosaccharide, generally short, is linked to serine o threonine (Preston *et al.*, 2013). In contrast, in all N-linked oligosaccharides, N-acetylglucosamine (GlcNAc) is linked to the amide nitrogen of asparagine, in the context of a tripeptide consensus sequence (Asn-X-Ser/Thr/Cys, where X can be any aminoacid, except proline) (Bause, 1983; Yan *et al.*, 2005). Typical N-linked oligosaccharides always contain mannose as well as GlcNAc and usually have several branches each terminating with negatively charged sialic acid residues. Most cytosolic and nuclear proteins are not glycosylated; the exceptions are several transcription factors and a protein localized into the nuclear-pore complex. The structure of N-glycans precursor is the same in eukaryotes: a branched oligosaccharide, containing three glucose (Glc), nine mannose (Man), and two GlcNAc molecules, described as $Glc_3Man_9(GlcNAc)_2$. Up to 25 enzymes located at the cytosol and endoplasmic reticulum (ER) are involved in the generation of this precursor. (Helenius, 1994; Trombetta, 2003) (Figure 8).

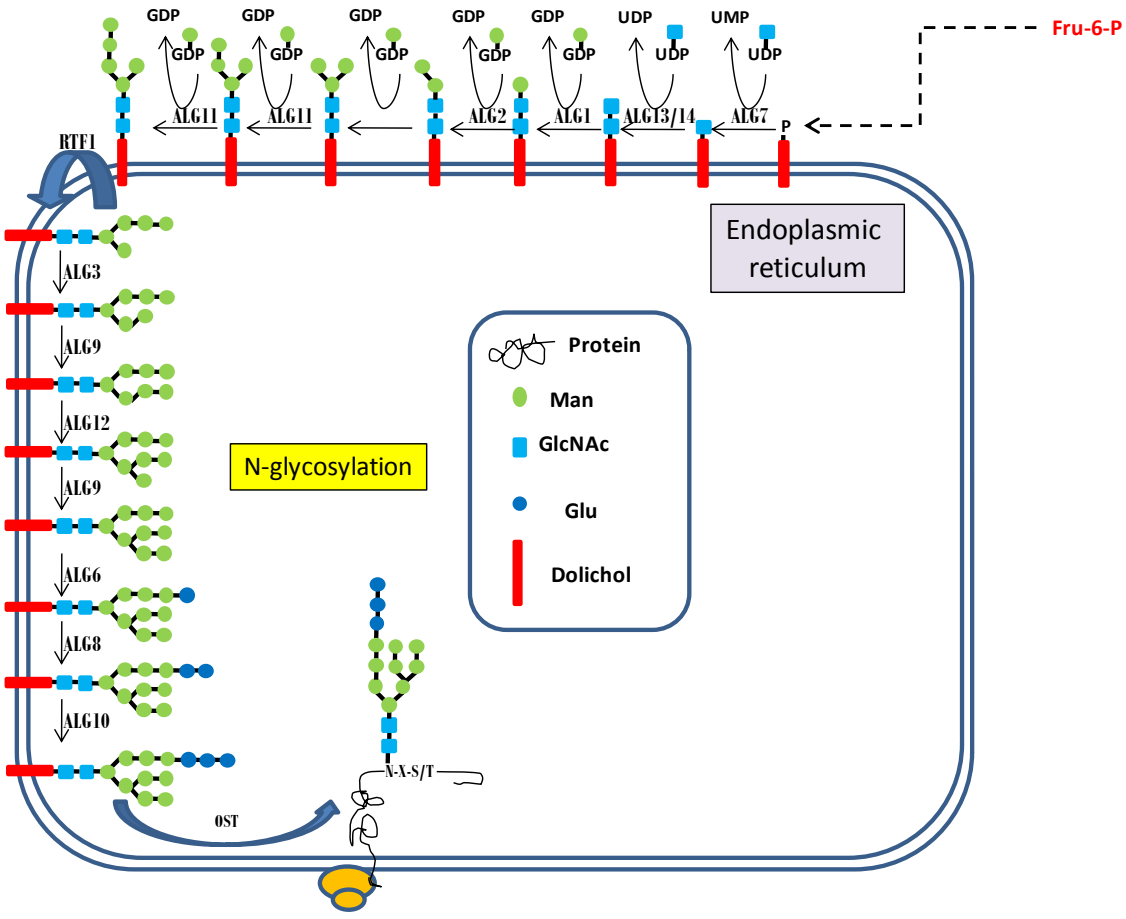


Figure 8. Scheme the N-glycosylation process in endoplasmic reticulum. The genes involved in each step are indicated. *GPI-anchor and GPI-anchored protein expression in PMM2-CDG patients. de la Morena-Barrio ME, Hernández-Caselles T, Corral J, García-López R, Martínez-Martínez I, Pérez-Dueñas B, Altisent C, Sevivas T, Kristensen SR, Guillén-Navarro E, Miñano A, Vicente V, Jaeken J, Lozano ML. Orphanet J Rare Dis. 2013*

The enzyme called oligosaccharyl transferase (OST) catalyzes the transfer of an oligosaccharyl moiety ($Glc_3Man_9GlcNAc_2$) from the dolichol-linked pyrophosphate donor to the side chain of asparagine within a consensus sequence (Asn-X-Ser/Thr or rarely Asn-X-Cys) (Mohorko *et al.*, 2014; Silberstein *et al.*, 1996).

Next, the protein moves to the Golgi apparatus. The enzymes localized into Golgi are those that catalyze additional modifications to the $Man_8(GlcNAc)_2$ oligosaccharide chains in glycoproteins produced in the rough endoplasmic reticulum. The sequential reactions add and remove specific sugar residues to yield a typical N-linked complex oligosaccharide in vertebrate cells (Figure 9). Five of the 14 residues of the N-glycan precursor are conserved in the structures of all N-linked oligosaccharides on secreted and membrane proteins (Lodish *et al.*, 2000). The figure 10 shows the final and typical N-glycan structure on mammalian glycoproteins. They are subclassified in three groups: high manose, hybrid and complex glycans (Preston *et al.*, 2013).

However, not all N-glycosylation consensus sequences become glycosylated; for instance, the rapid folding of a segment of a protein containing this sequence may prevent the transfer of $Glc_3Man_9(GlcNAc)_2$ to it.

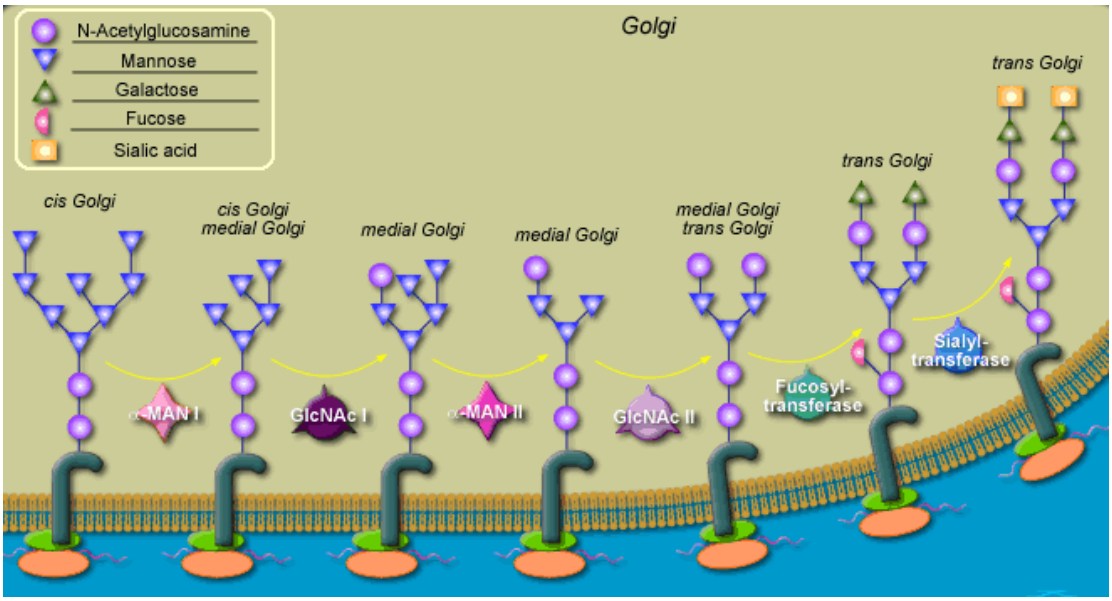


Figure 9. Representation of the N-glycosylation maturation with the initial core $Man_9GlcNAc_2$ sugar being formed in the ER and further modifications taking place in the Golgi apparatus. (http://worldofbiochemistry.blogspot.com.es/2011/08/metabolic-map-on-glycolsylation-process.html)

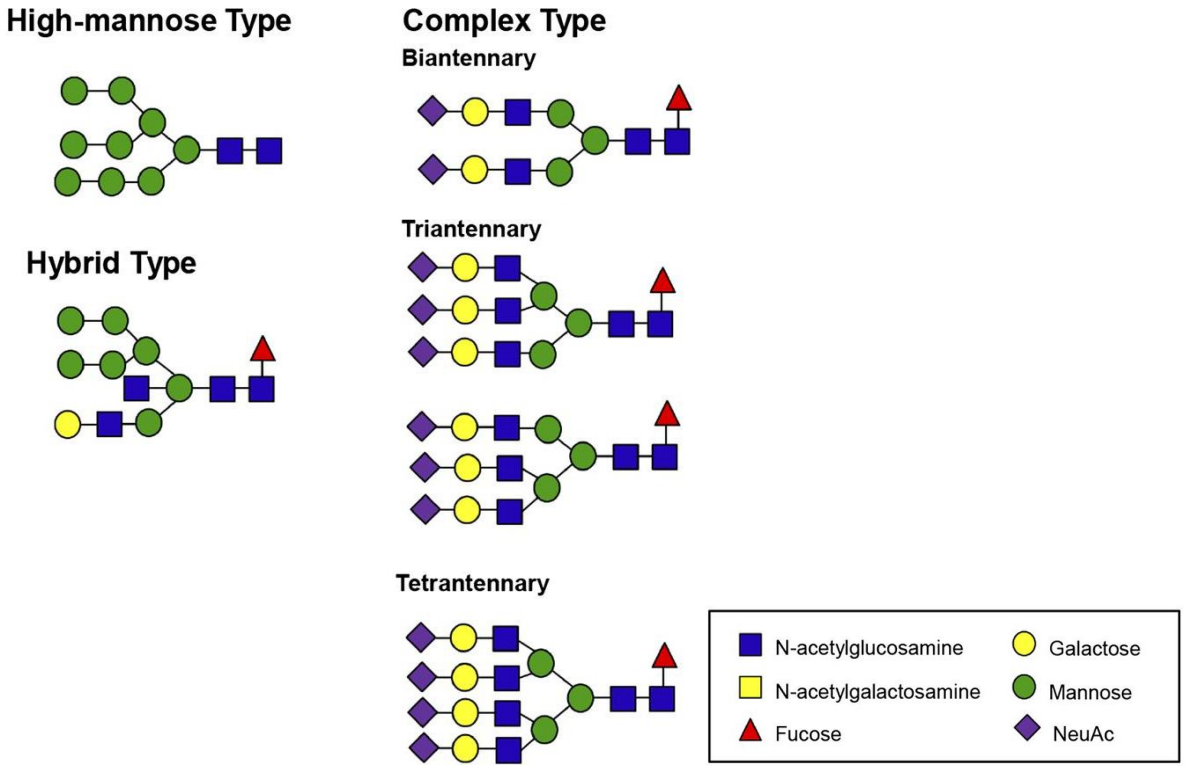


Figure 10. Representation of typical N-glycan structure on human glycoproteins. *Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities Preston RJ, Rawley O, Gleeson EM, O'Donnell JS. Blood. 2013*

N-glycosylation has been often used in protein drug development to increase the shelf-life and serum half-life of proteins, and to decrease their aggregation propensity, proteolytic susceptibility and even to determine the specificity of proteins (Brooks *et al.*, 2013; McGrath *et al.*, 2013; Mitra *et al.*, 2006; Narhi *et al.*, 1991; Ni Ainle *et al.*, 2011; Toyoda *et al.*, 2000). If the N-glycan could also increase protein thermodynamic stability, the pharmacokinetic properties of the protein could be enhanced (Elliott *et al.*, 2003; Golay *et al.*, 2013; Price *et al.*, 2012; Sinclair *et al.*, 2005). Therefore, there is a growing interest in the study of this post-translational modification, also in the hemostatic system. So, the physiopathological relevance of N- and O-glycosylation of platelet and coagulation proteins is an emerging field of research (Preston *et al.*, 2013).

3.1 N-glycosylation in antithrombin

Antithrombin also undergoes N-glycosylation during its synthesis in the hepatocytes. As indicated before, four consensus sequences are potentially N-glycosylated in antithrombin. However, the consensus sequence located at N135 is inefficiently glycosylated, which provokes the presence of two glycoforms in plasma: α- (with 4 N-glycans) and β-antithrombin (lacking of the N-glycan at N135) (Figure 11). This defective glycosylation has been

explained by the presence of a serine instead of a threonine in the consensus sequence of N135, unlike the rest of consensus N-glycosylation sites present on antithrombin (Picard *et al.*, 1995). However, the exact mechanism of this inefficient glycosylation is not fully understood. The consequences of the defective glycosylation are the lack of glycan in the β-glycoform, provoking differences such as a smaller molecular weight (56 kDa) and an increased isoelectric point (pI 5.625) compared with the α-glycoform (58 kDa and pI 5.375). However, the main difference between α and β-antithrombin concerns functional features. The absence of the N-glycan at N135, close to the HBS, modifies the heparin affinity, which is relevant for the anticoagulant role of this serpin. The lack of an N-glycan on β-antithrombin has two main consequences: higher heparin affinity (2-fold) and faster clearance rate in the circulation, than α-antithrombin (McCoy *et al.*, 2003; Ni *et al.*, 2000; Peterson *et al.*, 1985; Picard *et al.*, 1995; Turk *et al.*, 1997). In fact, there are 90% of α-antithrombin and 10% of β-antithrombin in plasma, despite they are synthetized in a similar proportion. All these features lead to consider that despite of being the minoritary gycoform, β-antithrombin could play relevant anticoagulant functions, particularly on the control of vascular thrombin after the injury of the vascular wall in aorta (Frebelius *et al.*, 1996; McCoy *et al.*, 2003; Witmer *et al.*, 1991).

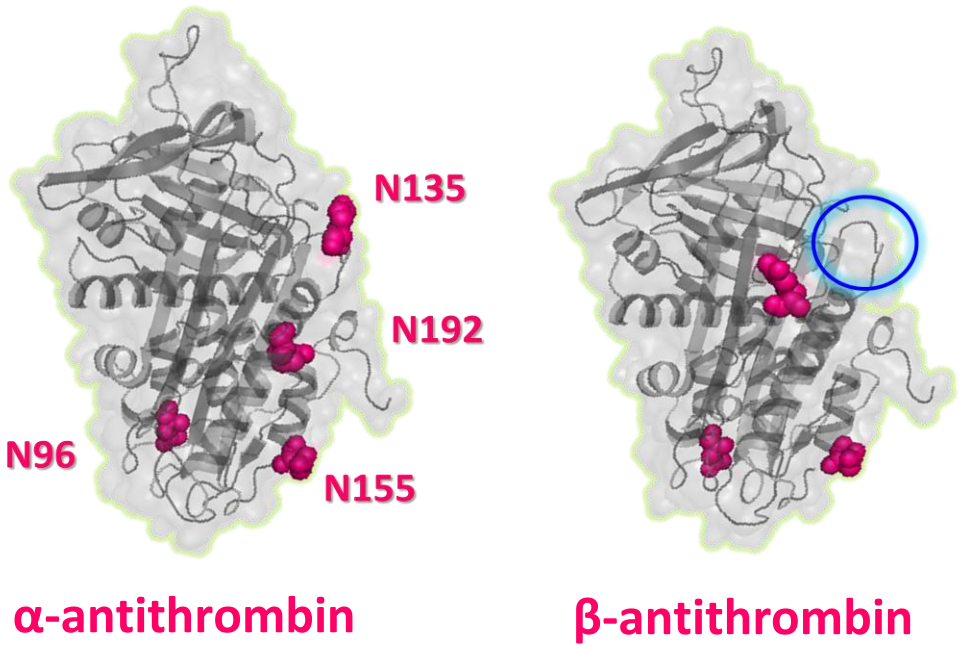


Figure 11. Ribbon diagram of native antithrombin (PDB code 1T1F). Spheres representation of the localization of N-glycosylation site in α (four glycans) and β-antithrombin (three glycans) (displayed in magenta). All structures were rendered in Pymol (www.pymol.org).

Moreover, antithrombin N-glycans with complex structure (Figure 10), but it does not usually present core-fucose on them. The fucosylation only occurs in CHO and BHK cells on one of the glycans of antithrombin. This explains the loss of heparin affinity of these recombinant antithrombin forms, as this component interferes with the heparin binding. (Garone *et al.*, 1996; Bjork *et al.*, 1992; Zettlmeissl *et al.*, 1989).

AIMS

The objective of the present PhD project was to deepen into the study of antithrombin, a key endogenous anticoagulant, through the analysis of natural mutants identified in patients with antithrombin deficiency, with the following specific aims:

1. **Characterization of new risk thrombotic factors associated with antithrombin deficiency.**

2. **Identification of new regions relevant for the antithrombin function.**

3. **Evaluation of the role and process of glycosylation in antithrombin.**

CHAPTERS

I. Compound heterozygosity involving Antithrombin Cambridge II (A384N) in antithrombin deficiency

Aim 1

Antithrombin is a key hemostatic anticoagulant serpin. More than 200 different mutations in *SERPINC1* have been identified in patients with thrombophilia, most of them in heterozygous state (HGMD: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1). In fact, homozygous deficiency is not compatible with life except for few homozygous or hemizygous patients with type II deficiency, mainly antithrombin Budapest III (L99F, numbering system according to the HGVS) and antithrombin Toyama (R47C), as these mutations present a milder thrombotic risk (Lane *et al.*, 1997; Martinez-Martinez *et al.*, 2012c). Antithrombin Cambridge II (A384N) is another type II deficiency and the most prevalent in normal population (0.2%, close to a low prevalent polymorphism) (Corral *et al.*, 2007). This antithrombin variant inefficiently inhibits thrombin (Mushunje *et al.*, 2003), increases the thrombin generation potential (Blajchman *et al.*, 1992), and moderately increases the risk of venous and arterial thrombosis (Blajchman *et al.*, 1992; Corral *et al.*, 2007; Mushunje *et al.*, 2003; Roldan *et al.*, 2009). Importantly, this mutation is not detected when evaluating anti-Xa activity or antithrombin antigen levels in plasma, the ordinary screening methods for antithrombin deficiency (Corral *et al.*, 2007). The potential hemostatic and clinical relevance of under diagnosing this defect in the general population might be significantly exacerbated among patients with antithrombin deficiency.

Functional, molecular, biochemical, and familial analysis of 93 unrelated Spanish and Portuguese patients with antithrombin deficiency, carried out as described elsewhere (Corral *et al.*, 2004; Martinez-Martinez *et al.*, 2010), revealed two cases with functional compound heterozygosity in the *SERPINC1* gene. In both cases, the A384N mutation (HGMD: http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1), (Caspers *et al.*, 2012; Luxembourg *et al.*, 2011), coexisted with a novel point mutation, previously undescribed. The first patient (P1) also carried the c.1190C>T mutation, leading to the S365L change that affects a highly conserved residue located at β-strand 5A. P1 is a 41-year old male, who developed an idiopathic pulmonary embolism and a deep venous thrombosis at the age of 38. Five out of seven maternal relatives with antithrombin deficiency also developed thrombotic episodes (Figure 1A). P1 had antithrombin antigen levels of 76% and disulfide-linked dimers in plasma (Figure 1A). The expression of this mutant in HEK-EBNA cells also revealed extracellular disulfide-linked dimers and moderate intracellular retention (data not shown). All these data support a conformational effect for the S365L mutation, which, according to recent studies (Martinez-Martinez *et al.*, 2012b) may contribute to explaining the severe thrombotic phenotype observed in carriers (Figure 1A).

The second patient (P2), a 33-year old woman who developed deep venous thrombosis at the age of 31, also carried the c.409 -2A>T, affecting the donor splicing signal of intron 2. P2 had a type I antithrombin deficiency according to her low antigen levels (52%) (Figure 1B).

No other thrombophilic factor was found in the family of either patient. Moreover, the anti-Xa activity of all carriers with antithrombin deficiency was around 50% (Figure 1). Antigen levels were similar in single or compound heterozygous (Figure 1). However, the anti-IIa activity, which was low in both patients, showed a significant reduction in compound heterozygous, reaching almost undetectable values in P2 (8%) (Figure 1). The normal range for activity was 70-120%.

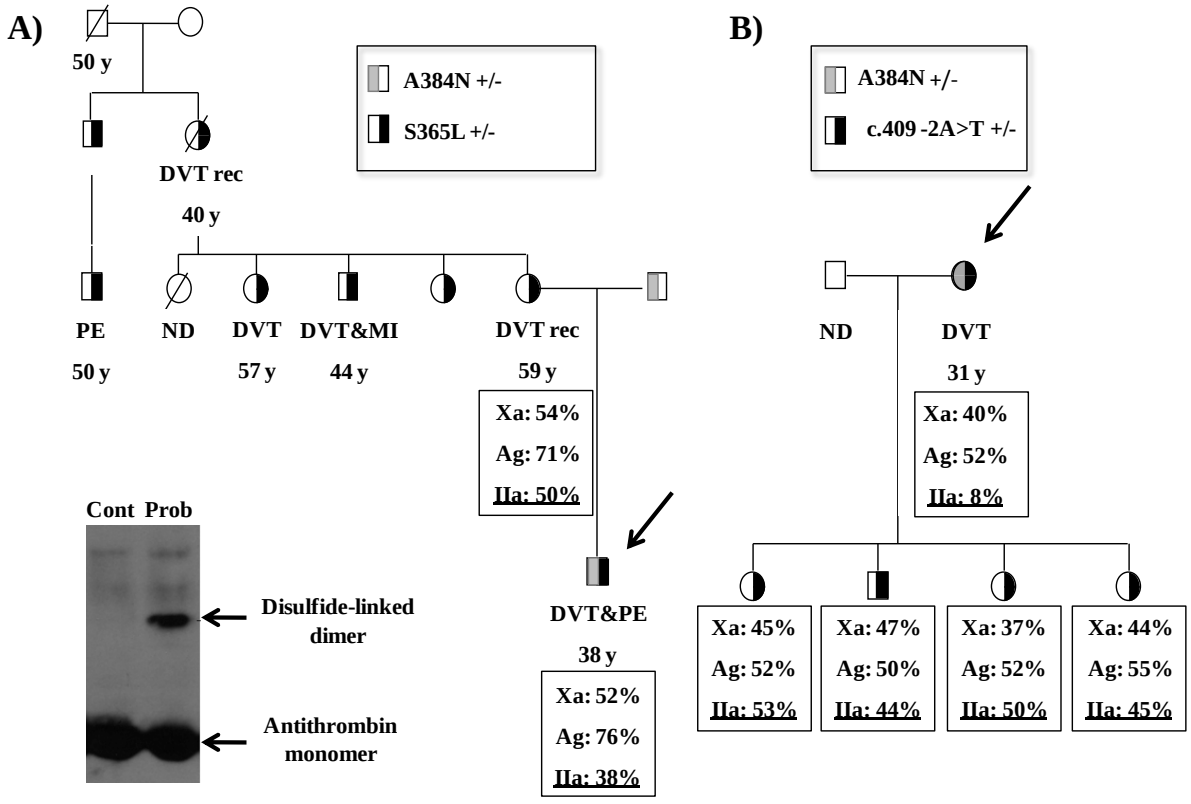


Figure 1. Clinical, laboratory and genetic data of members of families with compound heterozygosity of *SERPINC1*. **A)** Family 1: A384N & S365L. This figure also shows the identification by SDS under non-reducing conditions and western blot of disulfide-linked dimers of mutant antithrombin in plasma of a control (Cont) and the proband (Prob) with the S365L mutation. **B)** Family 2: A384N & c.409 -2A>T.

Inherited thrombophilic analysis aims to identify genetic risk factors for thrombosis to stratify subjects according to their thrombotic risk. The final purpose of these analyses is to facilitate the clinical management of carriers: to prevent recurrence in symptomatic patients or the first

event in asymptomatic carriers (Corral *et al.*, 2012). Antithrombin deficiency is one of the strongest risk factors for venous thrombosis, although there is considerable clinical heterogeneity (Lane *et al.*, 1997). Thus, some mutations, such as those affecting the heparin binding domain, cause a moderate risk (Martinez-Martinez *et al.*, 2012c). In contrast, other inactive mutants also have a gain-of-function, which, by different mechanisms, significantly reduce the anticoagulant capacity of carriers and explains their severe thrombotic profile (Corral *et al.*, 2012; Martinez-Martinez *et al.*, 2012b; Raja *et al.*, 2003). A combination of prothrombotic genetic risk factors may also increase the risk of thrombosis, as demonstrated in the case of common prothrombotic polymorphisms (de Haan *et al.*, 2012). For antithrombin deficiency, the rare combination with other severe thrombophilic defects, such as protein C or protein S deficiency associated with severe thrombosis (Jobin *et al.*, 1991). Similarly, the combination of antithrombin deficiency with factor V Leiden or prothrombin, which is not unusual, also increases the risk of thrombosis (van Boven *et al.*, 1996). Finally, homozygous or hemizygous patients are very rare, and their condition is always associated with antithrombin type II deficiencies (Martinez-Martinez *et al.*, 2012c). Compound heterozygosity might be an additional severe prothrombotic combination. Only two cases with compound heterozygosity in the *SERPINC1* gene have been described. One combines the Y166C mutation, which favors a failure in secretion, with a neutral M20T mutation (Perry *et al.*, 1995). The second was a real functional compound heterozygosity case combining an in-frame 9 bp deletion with a mild R47H mutation, leading to negligible heparin cofactor activity in compound heterozygous (Emmerich *et al.*, 1994b). Our study describes two cases of different functional compound heterozygosity in the *SERPINC1* gene, but both sharing the mild A384N mutation. Moreover, our study suggests that this combination is relatively frequent among patients with antithrombin deficiency in Spain (2/93: 2%), where the A384N mutation is present in 0.2% of the normal population (Corral *et al.*, 2007). Compound heterozygosity similar to that described here might be considered in other populations where Antithrombin Cambridge II is also a common defect in the general population (Tait *et al.*, 1994).

The presence of functional mutations in both alleles of the *SERPINC1* gene reduced the anticoagulant activity. In addition to an impaired anti-Xa activity, which is explained by the null activity of the allele affected by the strong mutation, the anti-IIa activity is severely reduced due to the presence of the A384N mutation in the other allele. These data suggest that compound heterozygosity involving the antithrombin Cambridge II mutation could increase the risk or severity of thrombosis. Further studies, such as thrombin generation assays and

larger family studies will be required to demonstrate this hypothesis in order to support a careful clinical management of these patients, including long term anticoagulation for symptomatic subjects and thromboprophylaxis under risk situations for asymptomatic carriers.

Finally, our results also have relevance for the accurate diagnosis of antithrombin deficiency, particularly in familial studies. Since the A384N is not detected by anti-Xa tests or antigen determination, and familial studies currently focused on detecting antithrombin deficiency by means of these tests or the identification of the index mutation by molecular methods, compound heterozygosity involving A384N might be underestimated. Actually, if the molecular analysis of the *SERPINC1* gene in family 1 had been performed in the mother of the proband (also with a severe thrombotic history) it is highly probable that the A384N mutation would never have been identified in the proband.

II. Identification of new residues in antithrombin involved in the reactive center loop insertion

Aim 2

1. Introduction

SERine Protease INhibitorS (serpins) are a super-family of proteins that controls proteases involved in inflammation, complement, coagulation and fibrinolytic pathways (Gils *et al.*, 1998). Inhibitory serpins exert the control over their target proteases by an unusual branched pathway with suicide substrate mechanism. The P1 residue or reactive site and flanking residues of the reactive center loop (RCL) of the serpin are recognized by the protease. Afterwards, the catalytic serine of protease attacks the reactive P1, resulting an acyl-intermediate complex in which the RCL is cleaved, but remains covalently linked to the serine. This cleavage triggers conformational changes in the serpin, thereby the cleaved RCL inserts into the central A sheet, leading to the dragged of the protease to the opposite end of the serpin (Huntington *et al.*, 2000; Olson *et al.*, 2010). In reactions between serpins and their target proteases the stoichiometry of inhibition is indistinguishable from one. Specific mutations on the RCL reduce the rate of loop insertion relative to deacylation, followed by inefficient inhibition of the protease. Therefore, the protease is released after the proteolytic attack and the serpin behaves as a substrate, provoking the increase of the stoichiometry of inhibition (SI > 1) (Audenaert *et al.*, 1994; Blajchman *et al.*, 1992; Caspers *et al.*, 2012; Lane *et al.*, 1997; Mushunje *et al.*, 2003; Olson *et al.*, 2010; Roldan *et al.*, 2009).

The aim of this study was to identify new regions and/or key residues for an efficient mechanism of inhibition outside of classical functional regions of antithrombin (RCL, HBS or C-sheet). Our study was initiated by selecting subjects that carried mutations causing a type II deficiency but they were localized outside of these functional domains. Further analysis with recombinant proteins confirmed that these variants affect the shift of the protease to the opposite end of the serpin, revealing a new region key for the last steps of the RCL insertion in the protease inhibition provoking an inefficient inhibitory mechanism.

2. Material and methods

2.1 Functional and genetic analysis of patients and family members

Our group has recruited during the last 10 years, 142 unrelated patients with antithrombin deficiency, mainly from different Spanish hospitals. The characterization of antithrombin deficiency fully described elsewhere (Aguila *et al.*, 2013; de la Morena-Barrio *et al.*, 2012a; Martinez-Martinez *et al.*, 2012c) included the following studies: i) Plasma antithrombin activity was determined by chromogenic assays in presence of heparin. Anti-FXa and anti-FIIa activity was evaluated; ii) antigen levels were determined by immunodifussion and ELISA; iii) heparin affinity of plasma antithrombin was evaluated by crossed-

immunoelectrophoresis; iv) electrophoretic features of plasma antithrombin assayed using native-PAGE (in presence and absence of 3M urea), and SDS-PAGE under reducing or non-reducing conditions; v) molecular analysis of *SERPINC1*, the gene encoding antithrombin. These studies included sequencing of the promoter region (1500 bp) and the 7 exons and flanking regions, as well as analysis of gross gene defects by MLPA.

2.2 Expression and purification of wild type and antithrombin variants

Site directed mutagenesis

Site directed mutagenesis was carried out using the Stratagene Quik Change Site-Directed Mutagenesis kit (Agilent, Madrid, Spain) or Quick Exchange kit (Invitrogen, Carlsbad, California, USA) and the appropriate primers. All mutations were confirmed by DNA sequencing.

Insect cells system

Recombinant antithrombin variants were constructed on an N135Q background to block glycosylation of Asn135 and thereby preventing the heparin binding heterogeneity that results from incomplete glycosylation at this site (Turk *et al.*, 1997). Variants were produced in baculovirus infected insect cells using the expression system from Invitrogen, as previously described by the manufacturer. Recombinant antithrombins were purified on a 5-ml Hi-Trap heparin-Sepharose column followed by ion exchange chromatography using a monoQ column, as previously described (Arocas *et al.*, 2001; Izaguirre *et al.*, 2003). Those fractions containing pure antithrombin were finally desalted and concentrations were determined by measuring the absorbance at 280 nm using an extinction coefficient of 37,700 M^{-1} cm^{-1} (Nordenman *et al.*, 1977). The purity of proteins was evaluated by 10% SDS-PAGE and coomasie staining. Finally, proteins were stored at -70 ºC.

HEK-EBNA system

We used the pCEP4-AT plasmid containing the cDNA sequence of human antithrombin, generously provided by Prof. J. Huntington (CIMR, Cambridge, UK). This plasmid, commonly used to produce recombinant antithrombin in Human Embryonic Kidney cells expressing the Epstein Barr Nuclear Antigen 1 (HEK-EBNA) cells, had the S137A mutation to only produce β-antithrombin (Picard *et al.*, 1995). HEK-EBNA cells were grown and transfected with the plasmid containing the mutation S137A/S365L as it has been described elsewhere (Martinez-Martinez *et al.*, 2010), and conditionated medium were harvested and stored at -70ºC.

2.3 Formation of covalent complexes

Recombinant antithrombin variants secreted to the conditioned medium of HEK-EBNA cells were incubated with FXa (8 µM) or FIIa (19 µM) (Enzyme Research Laboratories, Swansea, United Kingdom) for 15 min at 37 ºC. The reaction was carried out with and without 6.6 µM of unfractionated heparin (Mayne, Madrid, Spain), for 10 minutes. The final reactions were loaded in SDS-PAGE under non reducing conditions. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich, Madrid, Spain), followed by donkey anti-rabbit IgG–horseradish peroxidase conjugate (GE Healthcare, Barcelona, Spain), with detection via an ECL kit (GE Healthcare, Barcelona, Spain).

2.4 Assay conditions

Antithrombin-protease reaction stoichiometries and kinetics were done at 25 °C in either 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4, buffer for reactions with thrombin and factor Xa. The ionic strength of buffer was 0.15.

2.5 Stoichiometry of antithrombin-protease reactions

The stoichiometries for the reactions of purified variants with FIIa and FXa were determined as described previously (Olson *et al.*, 1993). Briefly, to fixed concentrations of 100 nM protease was added increasing concentrations of inhibitor to give molar ratios of inhibitor to protease of up to 2.8 in a final volume of 100 µl. Reactions were done both in the absence and presence of full-length heparin or pentasaccharide, which was fixed at an equimolar concentration with the protease. After incubating for times sufficient to complete the reaction (95%), 5 µl of the reaction mixture was added to 1 ml of substrate (100 µM S-2238 for FIIa or 100 µM Spectrozyme FXa for FXa), and the residual enzymatic activity was measured from the initial linear rate of change of absorbance at 405 nm. The decrease in protease activity with increasing molar ratio of inhibitor/protease was fit by linear regression to obtain the stoichiometry from the abscissa intercept.

2.6 Kinetics of antithrombin-protease reactions

Association rate constants for reactions of recombinant antithrombins with proteases in the absence or presence of pentasaccharide or full-length heparin were measured under pseudo-first order conditions by using at least a 10-fold molar excess of inhibitor over protease as in previous studies (Bedsted *et al.*, 2003; Izaguirre *et al.*, 2003). For reactions with all proteases in the absence of heparin and those reactions with proteases in the presence of pentasaccharide whose rate constants were 10^4 $M^{-1}s^{-1}$ or less (or in some cases as high as 10^5

$M^{-1}s^{-1}$), full reaction time courses of the loss of enzyme activity were obtained and fit by a single exponential function with a zero activity end point.

Pentasaccharide or heparin was present in these full time course experiments at levels that saturated antithrombin. Assays of residual enzyme activity were done by removing aliquots of the reaction at different times, diluting into appropriate chromogenic substrates, and measuring the initial rate of substrate hydrolysis as in previous studies (Yang *et al.*, 2002). Association rate constants for free antithrombin or antithrombin-pentasaccharide/heparin complex reactions were obtained from fitted exponential rate constants by dividing by the concentration of antithrombin. For all other reactions in the presence of pentasaccharide or full-length heparins, reactions were done for a fixed time as a function of the concentration of heparin, and the loss in enzyme activity was fit by a single exponential function with heparin concentration instead of time as the independent variable (Izaguirre *et al.*, 2003). The association rate constant for the reaction of the antithrombin-heparin complex was obtained from the fitted exponential rate constant by dividing by the fixed reaction time and by a factor that corrected for the fraction of heparin that was bound by antithrombin.

2.7 Determination of Denaturing Temperature

Thermal denaturation of antithrombin variants was monitored by changes in tryptophan fluorescence, with excitation wavelength at 280 nm and emission wavelength at 345 nm using the temperature controlled SLM-Instrument fluorometer. The rate of temperature change of 0.5 °C/min was used. Antithrombin concentration was 100 nanomolar in phosphate buffer pH 7.4 and *I*= 0.15. The data was fitted to the Van't Hoff equation:

$dG=-RT\ Ln(Fobs- Fn_0)/(Fd_0-Fobs) = dHm\ (1 – T/Tm)$.

dG= Gibb's free energy

R= gas constant

T=absolute temperature

Fobs = fluorescence observed.

Fn_0 = fluorescence coefficient of the native state

Fd_0 = fluorescence coefficient of the denatured state

dHm = enthalpy of denaturation

Fluorescence coefficients were determined from the linear parts of plots.

2.8 Heparin binding to antithrombin variants

Equilibrium binding of pentasaccharide to purified recombinant variants was evaluated by titrating pentasaccharide and monitoring the tryptophan fluorescence enhancement, as it has previously described (Olson et al., 1992). Because of the tight binding of recombinant antithrombin to heparin at physiologic ionic strength, binding studies were conducted in the phosphate buffer used for studying antithrombin-pentasaccharide reactions, but at a higher ionic strength (I = 0.35, attained by adjusting the NaCl concentration to 0.3 M) to allow more accurate measurements of K_D. Fluorescence titrations were computer-fit by the quadratic equilibrium binding equation assuming a 1:1 binding stoichiometry to determine values for K_D and the maximal fluorescence change (Olson et al., 1992).

3. Results

3.1 Identification of subjects with type II antithrombin deficiency. Clinical, genetic and analytical features

Among all the mutations identified in the 142 patients with antithrombin deficiency characterized by our laboratory, fifty-one associated with type II deficiency. Five of them, were not clearly located in key functional domains or regions of antithrombin: I207T; L340F; S349P; S365L and H369Y. We selected two cases, S365L and I207T as they are close in the tertiary structure of antithrombin despite of being distant in the primary sequence (Figure 1).

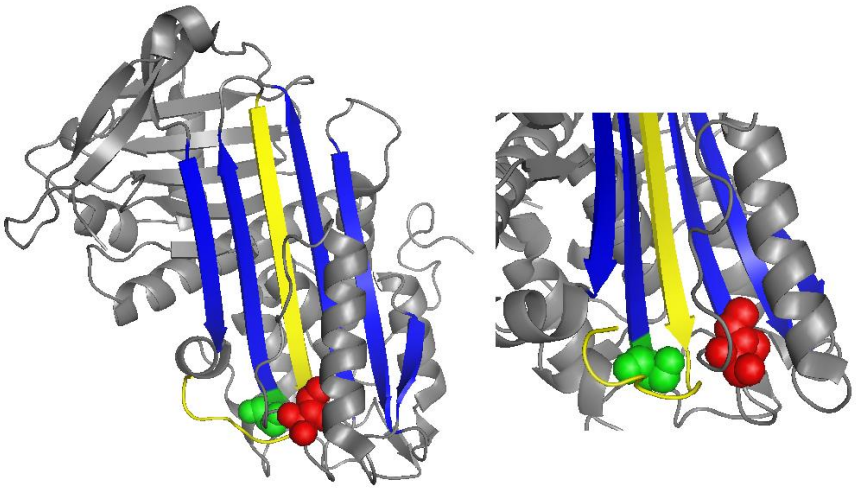


Figure 1. Ribbon diagram of latent antithrombin (PDB code 2BEH) showing the localization of I207 (red spheres) and S365 (green spheres) residues. The central A strands are shown in blue and the reactive center loop in yellow. All structures were rendered in Pymol (www.pymol.org).

Patients carrying these mutations did not have additional thrombophilic defects. The S365L carrier is a 59 year-old woman, who developed recurrent deep venous thrombosis (first episode at the age of 43). She showed heparin cofactor anti-Xa and anti-IIa activity values severely reduced, 54% and 50%, respectively, but slightly reduced antigen levels (71%). Moreover, a relatively high proportion of disulfide-linked dimers were detected in plasma by SDS-PAGE under non-reducing conditions (Figure 2).

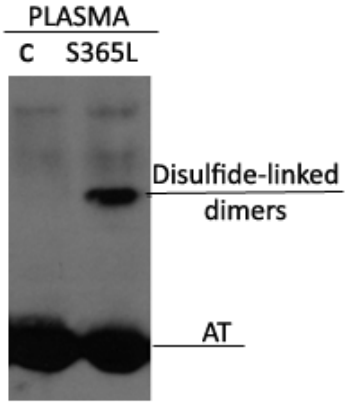


Figure 2. SDS-PAGE under non-reducing conditions for the detection of disulfide linked dimers of antithrombin in plasma samples of a healthy subject (C: control) and the patient carrying the S365L mutation (S365L). Antithrombin was detected by western blot. Native antithrombin (AT) and disulfide linked dimers are shown by arrows.

The I207T carrier is a 22 years-old asymptomatic woman, who presented anti-FXa values of 66%, while anti-FIIa and antigen levels were slightly reduced but within the normal range (86%, and 80%, respectively). No abnormal conformations of antithrombin was detected in plasma (data not shown). This mutation has not been described previously.

3.2 Formation of complexes by antithrombin variants

Recombinant expression of I207T and S365L in HEK-EBNA cells confirmed the type II deficiency observed in subjects carrying these mutations, since variants were correctly secreted to the conditioned medium (data not shown). Disulfide-linked dimers were observed in the conditioned medium of HEK-EBNA cells transfected with S365L mutant (Figure 3), supporting a conformational instability of this mutant, which explains why the protein precipitated during the concentration of purified sample. Due to the instability of S365L mutant, we evaluated its function using conditioned medium and analizing the formation of complexes with target proteases in SDS-PAGE under non-reducing conditions.

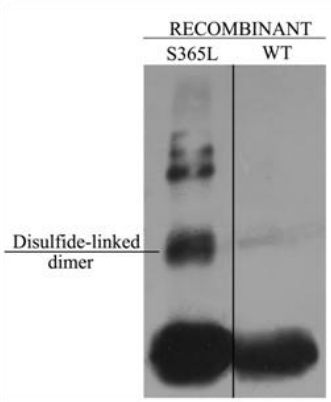


Figure 3. Expression of recombinant antithrombins in HEK-EBNA. Disulfide linked dimers of antithrombin are indicated by arrow.

The analysis of formation of covalent complexes of S365L mutant with FIIa and FXa showed that this variant did not form complexes and behaved as substrate with both proteases, according to the identification of cleaved antithrombin in SDS-PAGE under non-reducing conditions (Figure 4).

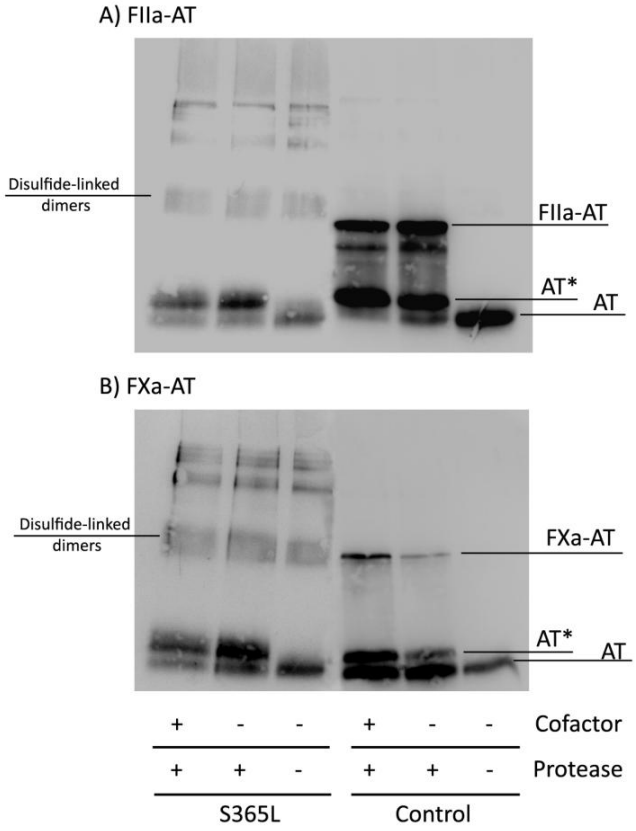


Figure 4. Formation of covalent complexes between antithrombin variants and target proteases. The control antithrombin (β-antithrombin) and variants were incubated with proteases: FIIa (A) or FXa (B) in absence and presence of unfractionated heparin or pentasaccharide, respectively. Conditioned medium of HEK-EBNA cells was used for these experiments and antithrombin was detected by western blot after SDS-PAGE non-reducing conditions. (Native antithrombin: AT, cleaved antithrombin: AT*, Thrombin-antithrombin complex: FIIa-AT, Factor Xa-antithrombin complex: FXa-AT).

3.3 Heparin affinity of I207T variant

Intrinsic fluorescence analysis revealed that purified I207T mutant presented increased heparin affinity. The dissociation constant and enhanced of fluorescence of this variant was lower (K_D = 39.1 ± 1.4 nM, $(\Delta F/F_0)\,x\,100$ = 32%) than control antithrombin (K_D = 62.4 ± 1.0 nM, $(\Delta F/F_0)\,x\,100$ = 38%).

3.4 Effects of the I207T mutation on the reactivity of antithrombin

Kinetic analysis confirmed that the I207T mutant was slightly affected in its reactivity with FIIa. Thus, the variant had a small increase in the stoichiometries, while the basal and activated rates were similar to control antithrombin (Table 1). However, this variant showed a high stoichiometry in the reaction with FXa and pentasaccharide (SI ~ 4). Moreover, the reactivity of this variant with FXa in presence of pentasaccharide was reduced 10-fold, compared with control antithrombin (Table 1).

Table 1. Association rate constants (Kapp x SI) and inhibition stoichiometries (SI) for control antithrombin (β-antithrombin) and I207T mutant reactions with FIIa and FXa. Values were measured as described in Material and Methods. H5, pentasaccharide; UFH, unfractionated heparin; FIIa, factor IIa; FXa, factor Xa; AT, antithrombin.

		FIIa		
		Kapp (M^{-1} s^{-1})	**SI**	**Kapp x SI**
Control	-	(1.1 ± 0.1) x 10^4	1.2 ± 0.1	(1.3 ± 0.2) x 10^4
	+UFH	(1.6 ± 0.1) x 10^7	1.7 ± 0.1	(2.7 ± 0.3) x 10^7
I207T	-	(3.8 ± 0.2) x 10^3	1.7 ± 0.1	(6.5 ± 0.7) x 10^3
	+UFH	(4.9 ± 0.1) x 10^6	2.9 ± 0.0	(1.4 ± 0.0) x 10^7
		FXa		
		Kapp (M^{-1} s^{-1})	**SI**	**Kapp x SI**
Control	-	(4.6 ± 0.4) x 10^3	1.1 ± 0.1	(5.0 ± 0.1) x 10^3
	+H5	(4.5 ± 0.5) x 10^5	1.5 ± 0.1	(6.9 ± 1.2) x 10^5
I207T	-	(2.8 ± 0.1) x 10^3	1.8 ± 0.1	(5.0 ± 0.0) x 10^3
	+H5	(1.7 ± 0.1) x 10^4	3.8 ± 0.2	(6.5 ± 0.1) x 10^4

3.5 Thermodynamic stability of I207T mutant

The thermal denaturation assay demonstrated that this variant had a structural instability since its melting temperature (*Tm*) in absence of heparin was 2.8ºC lower than control antithrombin. This difference was reduced in presence of heparin due to the structural stabilization and protection produced by the binding of this cofactor (Figure 5). The *Tm* of native and activated control antithrombin were 57.5 ºC and 58.8 ºC respectively, while that I207T mutant presented lower denaturation temperatures (54.7 ºC and 57.1 ºC) than control.

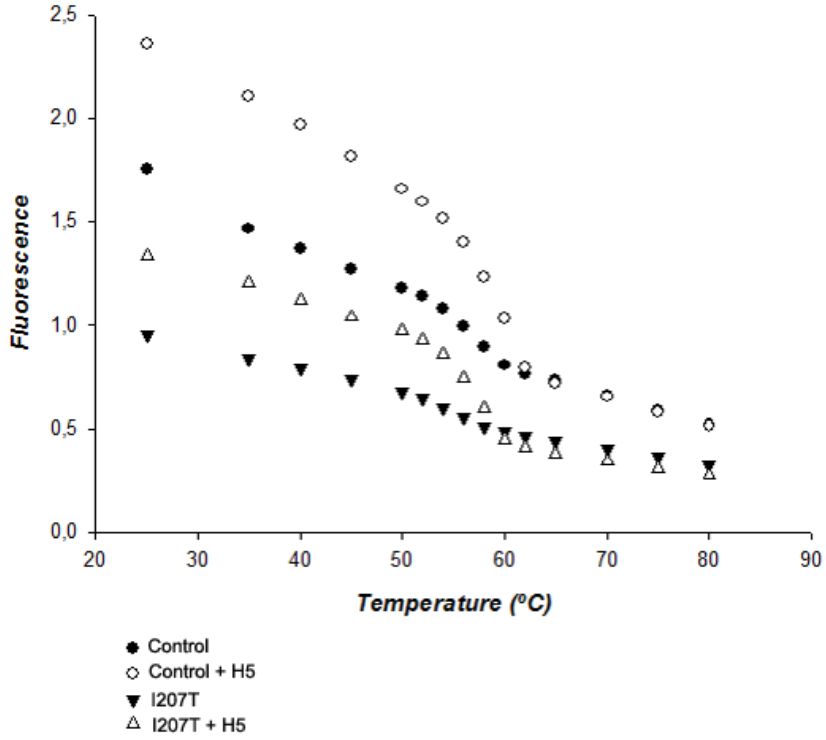


Figure 5. Thermal denaturation of control antithrombin, β-antithrombin (circles) and I207T mutant (triangles) in presence (white) or absence (black) of pentasaccharide (H5). The data was fitted to the Van't Hoff equation, dG=-RT Ln(Fobs- Fn_0)/(Fd_0-Fobs) = dHm (1 – T/Tm), as described in Material and Methods.

4. Discussion

Serpins share a common mechanism of protease inhibition and a molecular architecture based on a dominant five-stranded A β-sheet. The first step of this mechanism, the formation of the acyl-intermediate complex, has a critical importance because of its irreversible nature. The analysis of natural mutations identified in patients with antithrombin deficiency has assisted the identification of key functional domains or regions of this anticoagulant (Aguila *et al.*, 2014; Blajchman *et al.*, 1992; Corral *et al.*, 2007; Corral *et al.*, 2004; Koide *et al.*, 1984; Lane *et al.*, 1997; Raja *et al.*, 2003). Thus, mutations at the RCL, HBS and the C-sheet, responsible

for the three subtypes of type II deficiency, usually do not affect the folding and secretion of the variant, but impair the reactivity. Multiple evidences, from crystal and biochemical studies to mutant analysis have demonstrated that the RCL is crucial for the partitioning in favor of the stable covalent complex (Chuang *et al.*, 1999; Chuang *et al.*, 2001a; Martinez-Martinez *et al.*, 2012b). Specific mutations may interfere in the RCL insertion and the translocation of the protease to the opposite end of the serpin (Audenaert *et al.*, 1994; Blajchman *et al.*, 1992; Caspers *et al.*, 2012; Lane *et al.*, 1997; Mushunje *et al.*, 2003; Roldan *et al.*, 2009). In fact, mutations mostly located at the hinge region convert a proportion of this serpin into a substrate for the target protease (Hopkins *et al.*, 1993).

In this study, the analysis of natural mutations rendering variants with impaired reactivity has identified a new region, the opening for the inserted RCL to exit from the central A sheet, which is relevant for the last steps of the RCL insertion in the inhibition process. Mutations affecting these residues transform a significant fraction of the variant antithrombin into a substrate with the subsequent risk of thrombosis. In fact, the mutation S365L provokes that this variant completely behaves as substrate with both proteases, FIIa and FXa. A fraction of I207T mutant also behaves as a substrate for FXa.

Other residues located in other regions, but able to interfere with the RCL insertion might have the similar effect. Actually, the H369Y mutation also identified by our group in a patient with type II antithrombin deficiency and impaired reactivity also behaved as substrate with FIIa and FXa (Figure 6). This residue is located in the middle of the strand 5A and flanks the RCL when is incorporated in the central A sheet.

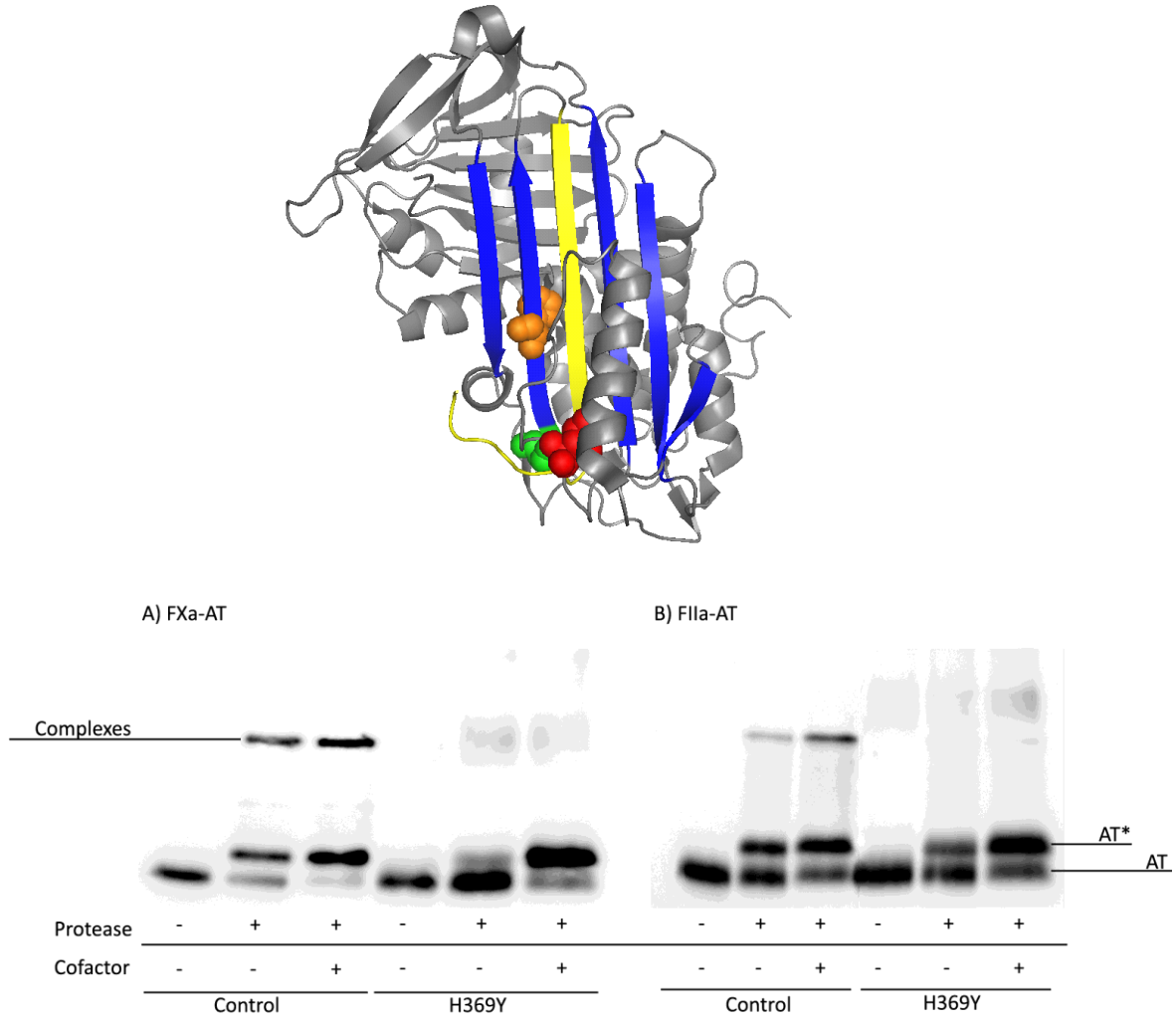


Figure 6. A) Ribbon diagram of latent antithrombin (PDB code 2BEH) showing the localization of I207 (red spheres), S365 (green spheres) and H369 (orange spheres) residues. The central A strands are shown in blue and the reactive center loop in yellow. The structure was rendered in Pymol (www.pymol.org). **B)** Formation of covalent complexes between H369Y mutant and target proteases. The control antithrombin (β-antithrombin) and the variant were incubated with proteases: FXa (A) or FIIa (B) in absence and presence of unfractionated heparin or pentasaccharide, respectively. Conditioned medium of HEK-EBNA cells was used for these experiments and antithrombin was detected by western blot after SDS-PAGE non-reducing conditions. (Native antithrombin: AT, cleaved antithrombin: AT* and complexes are shown by arrows).

It is important to point out that the patient carrying the H369Y mutation has increased levels of latent antithrombin in plasma (Figure 7).

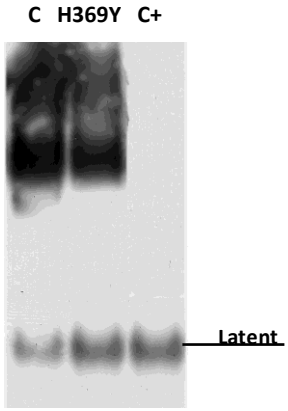


Figure 7. Native-PAGE with 3 M of urea for detection of latent antithrombin in plasma samples of a healthy subject (C), proband carrying the H369Y mutant (H369Y) and latent antithrombin (C+). Antithrombin was detected by western blot. Latent is shown by arrow.

This result, together with the disulfide-linked dimers observed in the plasma of the patient carrying the S365L mutation, and the reduced melting temperature of the T207 variant support the notion that conformational effects are probably involved in the deficiency observed in carriers of these mutations. Actually, S365, H369 and I207 residues are highly conserved among antithrombins of different species, which imply that they have structural relevance (Backovic *et al.*, 2002) and the conformational instability caused by mutations. The stressed native conformation of serpins, required for an efficient inhibitory mechanism, makes serpins vulnerable to environmental or genetic factors (Carrell *et al.*, 2003; Carrell *et al.*, 1985; Gettins, 2002; Hernandez-Espinosa *et al.*, 2007; Huntington *et al.*, 2000).

Finally, the data obtained with the I207T mutation has rendering some additional and interesting features. The inhibition of FIIa was very similar to control antithrombin. In contrast, the rates of FXa inhibition in presence of pentasaccharide was decreased 10-fold and the stoichiometry was higher than control (SI=3.8 vs 1.5). Therefore, I207T shows differential behavior depending on the target proteases. Thus, the mutation affects more severely the reactivity with FXa than with FIIa. This antithrombin variant resembles antithrombin Cambridge II (A384S), since its damaging effect is more severe in activated state, but with different protease (Corral *et al.*, 2007; Mushunje *et al.*, 2003). The stoichiometry of Cambridge II is weakly affected with FXa, but it is seriously perturbed with thrombin in the presence of bridging of full-length heparin due to an allosteric result (Corral *et al.*, 2007; Mushunje *et al.*, 2003). A potential explanation to these results is an interaction of I207 with residues of FXa during the dragging to the opposite end of the inhibitor. The change into threonine might remove this potential link or generate repulsive interactions, slowing the RCL internalization when FXa is covalently linked to it. Unfortunately, there are not available crystallized complexes between antithrombin and FXa in which the protease is already dragged to the opposite end in order to confirm this potential interaction.

In conclusion, we identified a new functional region for the anticoagulant activity of antithrombin. The S365 and I207 residues form an opening for the inserted RCL to exit from the central A sheet. Their mutations affect the reactivity of the variant antithrombins. Moreover, the interesting differential behavior of the I207T mutant with FIIa and FXa in activated state, also suggests new relevant interactions between serpin and proteases during the translocation that should be evaluated in future studies.

III. Role of C-sheet in the maturation of the N-glycans on antithrombin: functional relevance of pleiotropic mutations

Aim 2

1. Introduction

Hereditary antithrombin deficiency is a major genetic thrombotic risk factor, causing a 20-fold increased risk of venous thromboembolism (van Boven *et al.*, 1999). More than 230 different mutations have been described in the gene coding for antithrombin, which mostly occurs in a heterozygous state, with very few exceptions (Martinez-Martinez *et al.*, 2012c; van Boven *et al.*, 1999). They may cause type I or type II deficiencies. Depending on its localization and its functional consequences, type II deficiencies are further subclassified into three groups: i) type IIa: reactive center loop defects (RCL) ii) type IIb: heparin-binding site defects (HBS); and iii) type IIc: multiple or pleiotropic defects caused by mutations clustered at the s1C-s4C region (Figure 1). While in types IIa and IIb, the mechanism associated with the deficiency is well defined, in the type IIc, only one study has proposed that mutations in the 402-407 region may relay structural changes to the distal heparin binding site by perturbing the β-sheet and the core of the molecule (Lane *et al.*, 1992).

We characterized a pleiotropic mutations identified in a patient with venous thrombosis and antithrombin deficiency (K241E, Antithrombin Murcia) (Martinez-Martinez *et al.*, 2010). Two variants or glycoforms were observed in the plasma of the patient carrying this mutation and in a recombinant cell system expressing the mutant protein. One glycoform had an abnormal glycosylation (core-fucosylation) that explained the reduced heparin affinity (Garone *et al.*, 1996), while the second one, with normal glycosylation and heparin affinity, had impaired inhibitory capacity that was corrected by activation with heparin (Martinez-Martinez *et al.*, 2010). The aim of this study was to determine if other pleiotropic mutations share a similar mechanism to that described for antithrombin Murcia, as well as to define new potential functional regions in antithrombin.

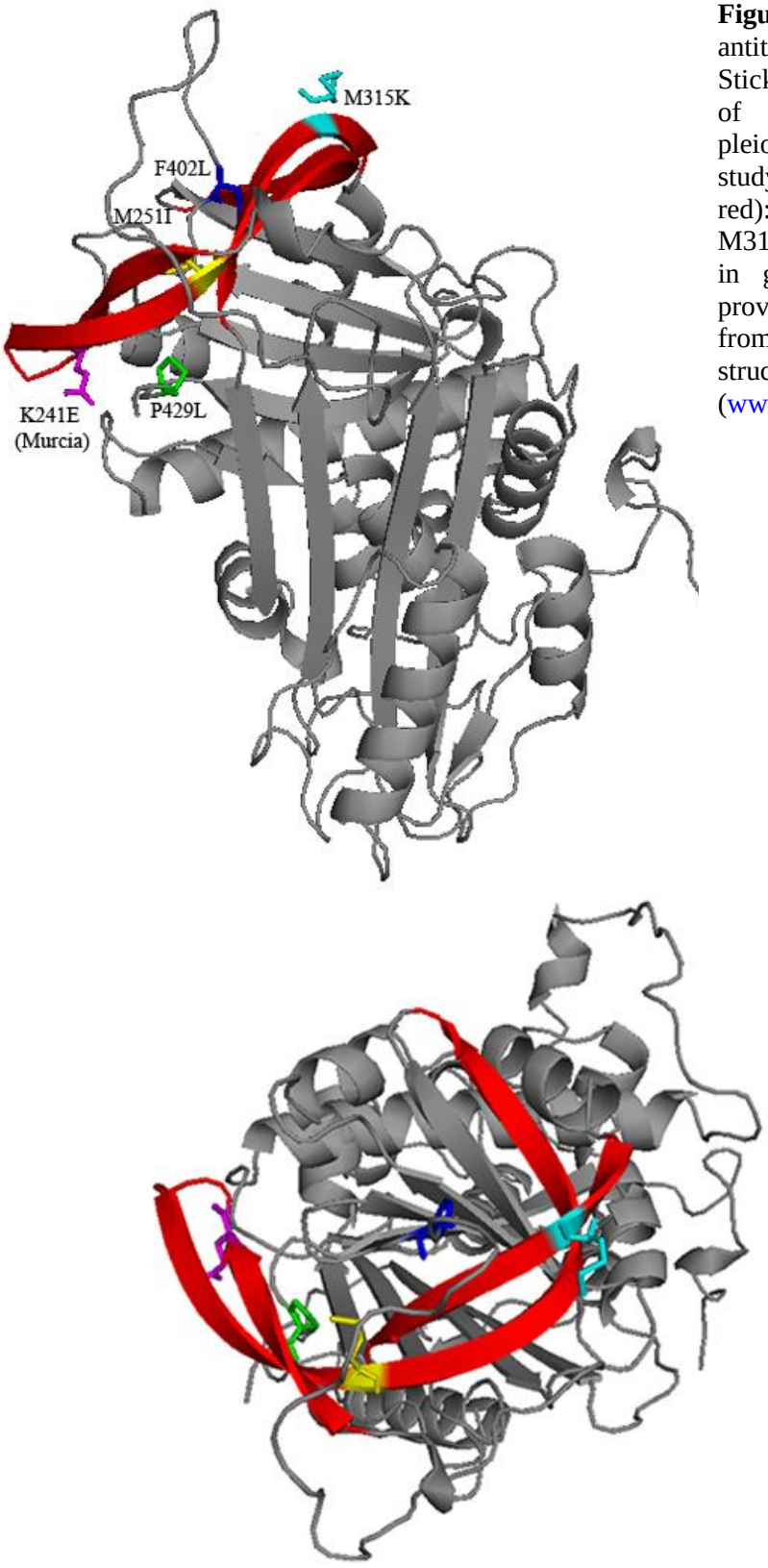


Figure 1. Ribbon diagram of native antithrombin (PDB code 1T1F). A) Stick representation of the localization of residue that presented natural pleiotropic mutantions selected in this study on the C-sheet (displayed in red): K241 in violet, M251 in yellow, M315 in cyan, F402 in blue and P429 in green. B) The ribbon diagram provides a close-up of C-sheet viewed from the top of the reactive loop. All structures were rendered in Pymol (www.pymol.org).

2. Material and Methods

2.1 Recombinant expression of wild-type and mutant antithrombins

Mutagenesis was performed using the Stratagene QuikChange Site-Directed Mutagenesis kit (Agilent, Madrid, Spain) and the appropriate primers to obtain the K241E, M251I, M315K, F402L and P429L mutants on the pCEP4/AT-S137A plasmid that produces β-glycoform of human antithrombin, generously provided by Dr. J Huntington (CIMR, Cambridge). Human Embryonic Kidney cells expressing the Epstein Barr Nuclear Antigen 1 (HEK-EBNA) were grown to 60% confluence at 37°C, 5% CO_2, in DMEM with GlutaMAX-I medium (Invitrogen, Barcelona, Spain) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Madrid, Spain). We transfected 200 µg/mL of wild-type (WT) and it with the different mutations for 30 minutes in OptiMEM with LTX (Invitrogen, Barcelona, Spain), as suggested by the manufacturer. After 24 hours, the cells were washed with PBS and exchanged into CD-CHO medium (Invitrogen, Barcelona, Spain) supplemented with 4mM L-glutamine and 0.25 mg/mL Geneticin (Invitrogen, Barcelona, Spain). Cells were grown at 37ºC for 10 days. The media was harvested and replaced by fresh medium every 2 days.

2.2 Secretion and intracellular retention of antithrombin variants

Cells were transfected as described previously. After, 24 hours of incubation with CDCHO, the conditioned medium was withdrawn and stored at -70ºC. Then, cells were extensively washed with sterile PBS and then lysated with 50 µl of lysis buffer (10 mM TrisHCl, 0.5 mM DTT, 0.035% SDS, 1mM EGTA, 50 mM sodium fluoride, 50 µM sodium orthovanadate, 5 mM benzamidine and 20 mM phenylmethylsulphonyl fluoride) and stored at -70ºC. Intracellular and secreted antithrombins were evaluated by western blotting in 8% SDS-PAGE under reducing conditions. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich, Madrid, Spain), followed by donkey anti-rabbit IgG–horseradish peroxidase conjugate (GE Healthcare, Barcelona, Spain), with detection *via* an ECL kit (Amersham Biosciences, Piscataway, NJ). Monoclonal anti β-actin (Sigma-Aldrich, Madrid, Spain) was used to check sample loading.

2.3 Protein purification and electrophoretic evaluation

Recombinant proteins were purified by heparin affinity chromatography on 5 mL HiTrap Heparin columns (GE Healthcare, Barcelona, Spain), using an ÄKTA Purifier (GE Healthcare, Barcelona, Spain) in 50 mM Tris-HCl, pH 7.4 buffer where a gradient from 0 to 3 M NaCl was applied to elute the proteins. Next, different variants of antithrombin were

purified by anion exchange chromatography on 1 mL HiTrap Q columns (GE Healthcare, Barcelona, Spain). Those fractions containing pure antithrombin were finally desalted, concentrated and stored at -70 ºC. Purity and separation of different variants or glycoforms were evaluated by 10 % SDS-PAGE under reducing conditions and immunoblotting, as indicated elsewhere (Corral *et al.*, 2007). SDS-PAGE under non-reducing conditions was run to detect disulfide-linked dimers and urea-PAGE (6 mol/L urea) was performed to detect latent forms (Corral *et al.*, 2007; Mushunje *et al.*, 2004).

2.4 Antithrombin activity

Antithrombin activity was determined using chromogenic anti-FXa and anti-FIIa assays in absence and presence of heparin (HemosIL Liquid Antithrombin, Instrumentation Laboratory, Kirchheim, Germany). Protein concentration was calculated by gel densitometry and bicinchoninic acid protein (BCA) assay.

2.5 Thrombin-antithrombin complex formation

Formation of thrombin-antithrombin complexes (TAT) was evaluated by incubation of 5.4 µM antithrombin with 19 µM thrombin (Calbiochem, Millipore, Madrid, Spain) at 37ºC. Aliquots were withdrawn at different intervals of time (10 min and 1 h). The reaction was carried out with and without previous incubation of antithrombin with 6.6 µM heparin for 10 min. These samples were evaluated by SDS-PAGE as indicated previously.

2.6 Interaction with neutrophil elastase

Purified glycoforms of all pleiotropic mutants were treated with 8.5 µM of human neutrophil elastase (HNE) (Calbiochem, Millipore, Madrid, Spain) for 30 min at 37ºC, in presence of 6.6 µM of unfractionated heparin. The effectiveness of the reaction was evaluated by SDS-PAGE under non-reducing conditions, which allows the detection of cleaved antithrombin (Corral *et al.*, 2007; Mushunje *et al.*, 2004).

2.7 N-glycosidase F digestion

Purified antithrombin variants were treated with N-glycosidase F (Roche Diagnostics GmbH, Mannheim, Germany), which required a previous denaturing step (5 min at 100ºC in 150 mM sodium phosphate buffer, pH 7.4 and 10% NP-40). Afterwards, samples were chilled on ice and digested with 0.6 U N-glycosidase F by incubation at 37ºC over night. Samples were resolved by 10% SDS-PAGE under reducing conditions and detected by silver staining.

2.8 Intrinsic fluorescence studies

Equilibrium dissociation constant (K_D) for the antithrombin-heparin interaction was determined essentially as described previously (Langdown *et al.*, 2009). Briefly, change in intrinsic fluorescence of antithrombin (25 nM) upon titration of the unfractionated heparin was monitored at 340 nm on Hitachi F4500 spectrofluorometer, with excitation at 280 nm and using bandwidths of 3.5 nm for both excitation and emission. All titrations were carried out at room temperature (25 ºC) under physiological ionic strength (I = 0.15) in 20 mM $NaPO_4$, 100 mM NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4. Fluorescence emission intensity was taken as the average of 100 measurements recorded at 1-s intervals for each addition of heparin. Data were fitted as previously described (Langdown *et al.*, 2009).

2.9 MALDI-TOF-MS analysis

A solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 g/L) in acetonitrile (ACN)/water/trifluoroacetic acid (TFA) (50:50:0.1 by vol.) was chosen as matrix for protein analysis. Experiments were carried out on a 4800 Plus MALDI TOF/TOF™ Analyzer (ABSciex, Madrid, Spain), equipped diode-pumped, solid-state laser. Recorded data were processed with Data Explorer™ Software (Applied Biosystems, Life Technologies, Madrid, Spain). Released carbohydrates (N-glycosidase F digestion, vide infra) were analysed using the same instrument and 2,5 dihydroxibenzoic acid (20 g/L in methanol/water (1:1)) as matrix.

2.10 Glycan profiling

Glycans were released from the antithrombin and variants following the protocol established earlier (Martinez-Martinez *et al.*, 2010). Briefly, glycoproteins variants were lyophilized and resuspended in the correct deglycosylation buffer (50 mM phosphate, pH 7.3 containing 0.5% SDS). Then, 1 U PNGase F were added and samples were incubated at 37 °C overnight with continuous agitation (750 rpm). Glycans were separated from the deglycosylated protein by filtration over 10 kDa centrifugal filters and subsequently purified over graphitized carbon. Following lyophilisation, carbohydrates were labelled with 2-amino benzamide following the established protocol (Gutierrez Gallego *et al.*, 2004). Normal phase chromatography of 2-aminobenzamide (2-AB) N-linked glycans were carried on ACQUITY Ultra-Performance Liquid Chromatography System at Ethylene Bridged Hybrid particle of 1.7 μm (BEH UPLC) with an Agilent 1100 HPLC equipped with a fluorescence detector (1100 Agilent fluorescence module): excitation λ=330 nm and emission λ=420 nm (Agilent, Madrid, Spain).

The system was calibrated in glucose units using a 2-aminobenzamide-labeled dextran hydrolysate. The total running time was 125 min. Samples were reanalyzed following exoglycosidase digestions, essentially α2-R sialidase and α2-R sialidase with β1-4 galactosidase, essentially as described before (Llop *et al.*, 2007).

3. Results

3.1 Pleiotropic mutations selected

We selected pleiotropic mutations identified in patients with antithrombin deficiency from available data bases (www.hgmd.org) (Emmerich *et al.*, 1994a; Lane *et al.*, 1993; Lane *et al.*, 1992; Martinez-Martinez *et al.*, 2010; Millar *et al.*, 1994; Olds *et al.*, 1992; Picard *et al.*, 2006): K241E, M251I, M315K, F402L, and P429L. Each one of these mutations was located at one of the strands of the C-sheet (s1C-s4C), except for the P429L mutation that was located at the C-terminal end of antithrombin. The structural localization of these mutations is depicted in Figure 1. The pleiotropic effect of these mutations was supported by the decreased anticoagulant activity, moderately reduced antigen level and reduced heparin affinity (Table 1).

Table 1. Clinical and laboratory data reported in the bibliography of patients with antithrombin deficiency carrying selected pleiotropic mutations. The structural localization of the mutated residue and the conservation among the serpin superfamily is also indicated. Abbreviations: NC: Not conserved. HC: Highly conserved. AT- activity: antithrombin anti-FIIa or anti-FXa activity with heparin; Ag: antigen level; TE: thrombotic event; FTE: age of first thrombotic event, DVT: deep venous thrombosis, PE: pulmonary embolism, r DVT: recurrent deep venous thrombosis; MVT: mesenteric venous thrombosis

Mutation	AT-activity	Ag	TE	FTE	Structural localization	Reference
M315K	63%	78%	DVT 35 y	35 y	s2C	Picard et al.,2006 Human mut. (14)
	71%	95%	Asymptomatic	no	N.C	
F402L	56%	73%	r DVT	55 y	s1C	Lane et al, 1992 J. Clin. Invest.(11)
	55%	75%	r DVT	17 y	Conserved	
	71%	73%	Asymptomatic			
	41%	73%	r DVT/PE	25 y		Emmerich et al., 1994 Thromb. Research (1)
	48%	65%	DVT	17 y		
M251I	52%	73%	MVT		s3C	Millar et al., 1994 Hum. Genet. (12)
					H.C	
P429L	36%		Thromboembolic episodes		C-terminal	Olds et al., 1992 Blood (13)
(Homozygous)					H.C	
K241E	60%	77%	DVT	44 y	s4C	Martínez-Martínez et al., 2010 Haematologica (4)
					N.C	

3.2 Secretion and intracellular retention

Recombinant antithrombin mutants presented similar intracellular retention and secretion to the conditioned medium than wild-type antithrombin, which agrees with the classification of these mutations as causing a type II deficiency in the patients (Figure 2 A).

3.3 Purification and identification of glycoforms

Purification of the different mutants from conditioned media was performed by heparin affinity chromatography. SDS-PAGE under reducing conditions and western blot of eluting fractions revealed two antithrombin forms or variants (V1 and V2) with different mobility that eluted at different salt concentrations in all mutants (data not shown), except for M315K, that only presented one variant. The two variants for each mutation were then purified and isolated by anion exchange chromatography at pH 7.4 and the purity of the different variants was evaluated by 10% SDS-PAGE under reducing conditions (Figure 3). Unfortunately, V2 from

P429L was not separated completely from V1 in enough amounts for all assays, even using different anion exchange strategies of purification (Figure 3)**.**

The form with higher heparin affinity (V2) always eluted at similar fractions and presented similar electrophoretic mobility than WT antithrombin. In contrast, the variant 1 (V1), which eluted at low salt concentrations, displayed less electrophoretic mobility than WT antithrombin. Interestingly, the mobility of V1 was slightly different depending on the mutation (Figure 2 B, 3). The treatment with N-glycosidase F equalized the electrophoretic mobility of all variants (Figure 2 B), which suggested that differences between V1 and V2, and among V1 from different mutations, were caused by differential N-glycosylation composition (Figure 2 B).

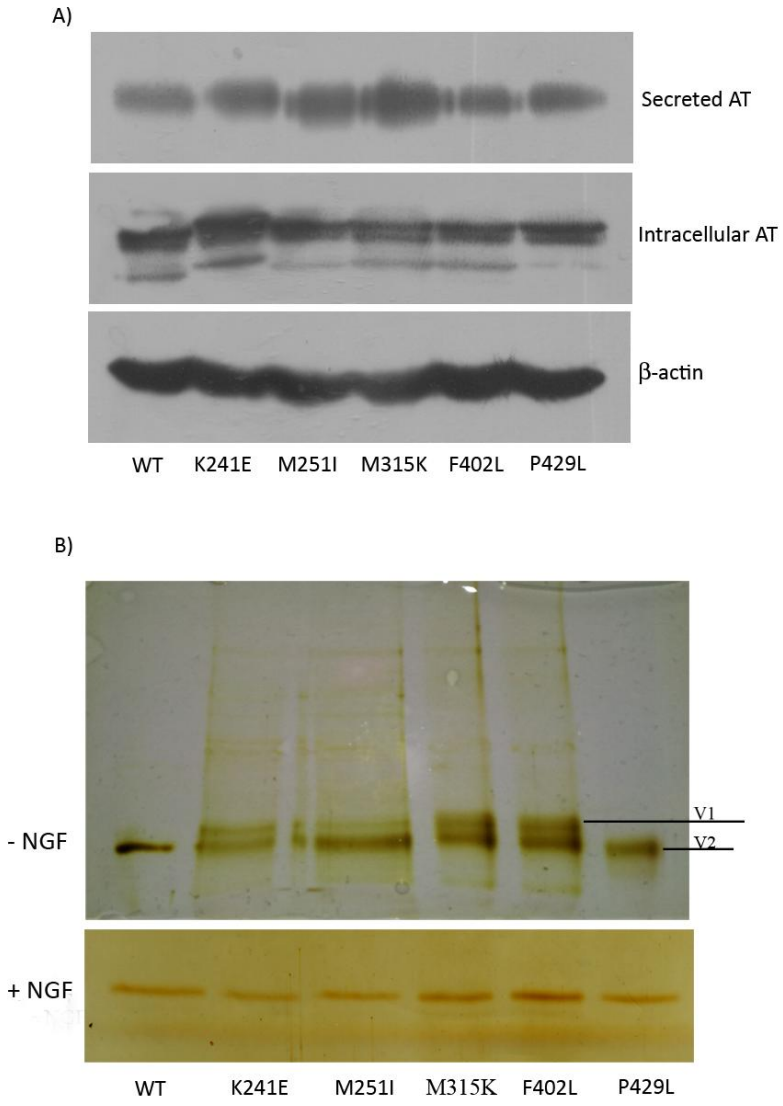


Figure 2. Electrophoretic separation by SDS-PAGE under reducing conditions of recombinant antithrombins produced and secreted by HEK-EBNA and purified antithrombin variants. **A)** Western blotting of antithrombin mutants secreted by the cells 24 h after transfection. Intracellular antithrombin and β-actin are also evaluated. As indicated in the Material and Methods section, all recombinant

antithrombins were generated in the β context (S137A) to reduce the glycosylation heterogeneity. **B)** Purified antithrombin variants untreated with N-glycosidase F (-NGF) and treated with N-glycosidase F (+NGF). The variants were purified and then mixed to easy distinguish the different mobility. Recombinant proteins were detected by silver staining. In addition to the selected pleiotropic mutations, we also used wild-type (S137A, WT) antithrombin as a control.

Figure 3. SDS-PAGE gels under reducing conditions of purified antithrombin variants. M315K mutant only presented one variant (V2). The variant 1 (V1) from the P429L mutant was not separated completely from variant 2 (V2). Recombinant proteins were detected by silver staining. WT: wild-type antithrombin.

3.4 MALDI-TOF-MS and glycosylation analysis

The MALDI-TOF studies showed that the deglycosylated variants had very similar masses (approximately 49 kDa), which indicates that the differences in the electrophoretic mobility between V1 and V2 should be caused by a different N-glycan composition (Figure 4).

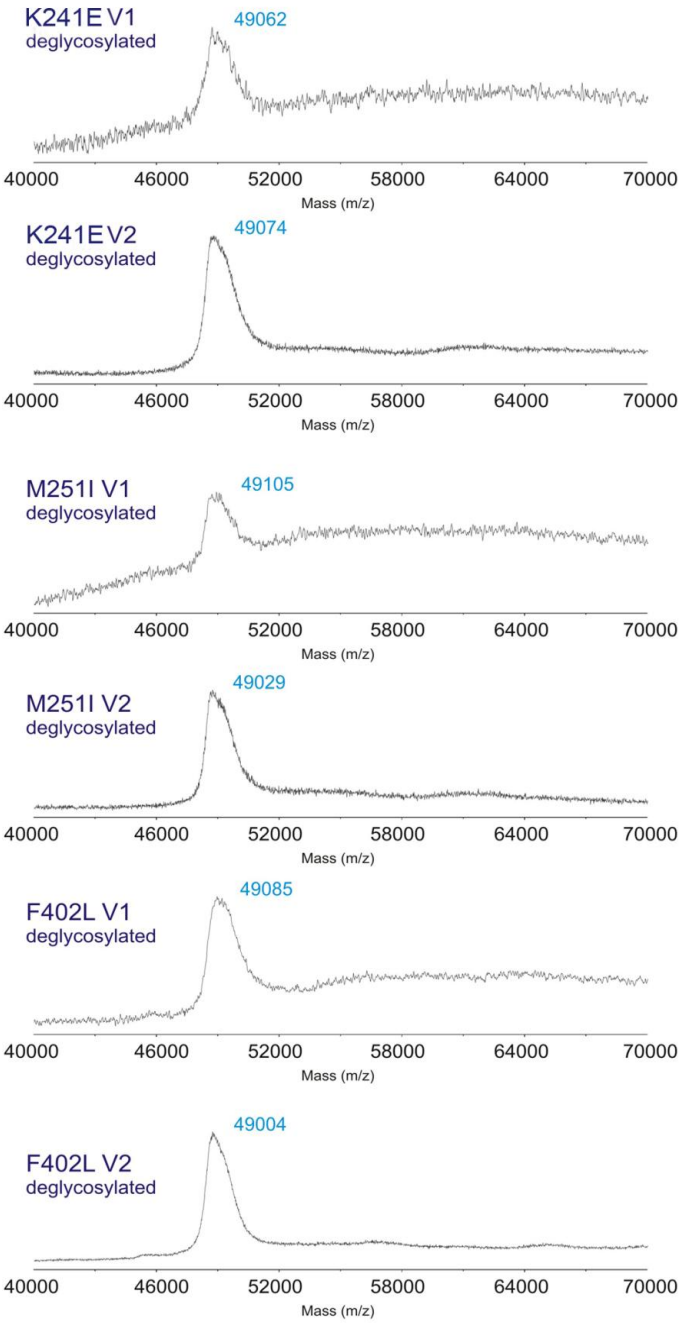


Figure 4. Mass spectra by MALDI-TOF-MS analysis of the purified variants (V1 and V2) of pleiotropic mutants after treatment with N-glycosidase F.

Advanced glycomic analysis with exoglycosidase treatment and mass determination was also done with V1 and V2 glycoforms of all pleiotropic mutations, except for P429L due to the inefficient chromatographic separation of the two glycoforms of this variant. Unfortunately, the low amount of purified variants did not allow us to obtain conclusive results, except for M251I. Interestingly, most of the V1 glycoforms of M251I contain at least one fucose residue

(Figure 5). Therefore, this is the first result that directly identifies a fucose in the abnormal glycan of a variant of antithrombin caused by a point mutation.

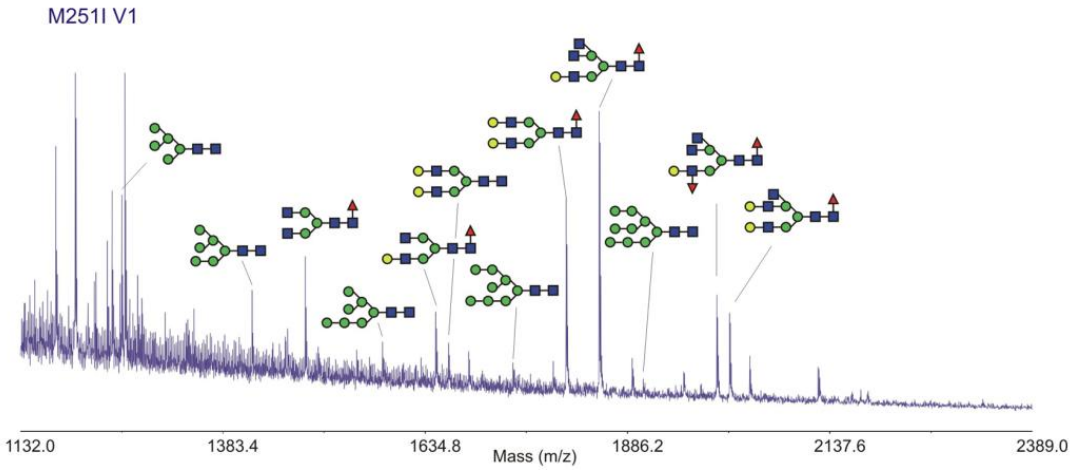


Figure 5. Spectra of glycans composition of the variant 1 (V1) with M251I mutation obtained by mass spectrometry after digestion with N-glycosidase F. The red triangle represent fucose, the yellow cicle represents mannose, the green circle represents glucose and the blue square represents N-acetylglucosamine.

3.5 Heparin affinity and inhibitory activity

The heparin affinity of the different variants was evaluated by intrinsic fluorescence assay. The equilibrium dissociation constants (K_D) showed that all V1 presented a reduced heparin affinity compared with the heparin affinity of WT antithrombin and V2 (Table 2). Thus, V1 from K241E and M251I showed a 4.5- and 5-fold reduction in the heparin affinity compared with V2 and WT, respectively, while the reduction was moderate for V1 from F402L (3-fold) (Table 2, Figure 6). However, there were not differences in the fluorescence increase between V1 and V2 of each one of the mutants evaluated (Table 2).

Table 2. Equilibrium dissociation constants for the binding of unfractionated heparin by antithrombin (AT). Dissociation constants (K_D) and maximal fluorescence increase were measured by global fitting of fluorescence titrations with unfractionated heparin as described in Materials and Methods section.

Recombinants AT	***KD* (nM)**	**(ΔF max/Fo) x100**
WT	37.6 ± 3.1	38.3
K241E V1	257.6 ± 2.8	41.0
K241E V2	55.7 ± 4.5	42.6
M251I V1	290.0 ± 2.4	38.4
M251I V2	50.0 ± 5.3	38.7
M315K	52.9 ± 5.6	41.3
F402L V1	151.9 ± 1.5	41.6
F402L V2	50.7 ± 1.0	37.5
P429L V1+V2	72.9 ± 4.0	41.0
P429L V2	48.2 ± 2.2	41.3

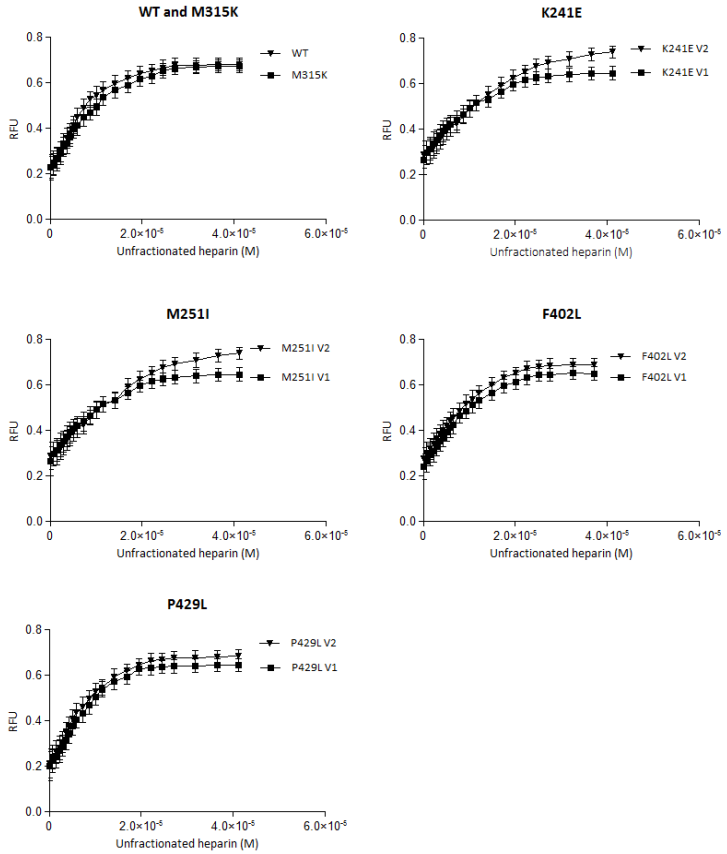


Figure 6. Heparin binding titrations. The intrinsic fluorescence change upon unfractionated heparin addition was followed for antithrombin wild type or variants 2 (V2) (black inverted triangle); and for variants 1 (V1) and M315K (black square) of all pleiotropic mutants.

On the other hand, the anti-FXa and anti-FIIa inhibitory activity of the different mutants was assessed by chromogenic methods using the conditioned medium of HEK-EBNA cells. Both variants from all mutants showed very low inhibitory activity of both proteases in the absence of heparin (Figure 7). Only, the M315K mutant (where only one form was detected) presented a slightly impaired inhibitory activity that was fully recovered by activation with heparin (Figure 7). Furthermore, the rest of antithrombin mutants partially recovered the inhibitory activity when they were activated by heparin, excluding F402L, which had negligible activity even in the presence of heparin (Figure 7).

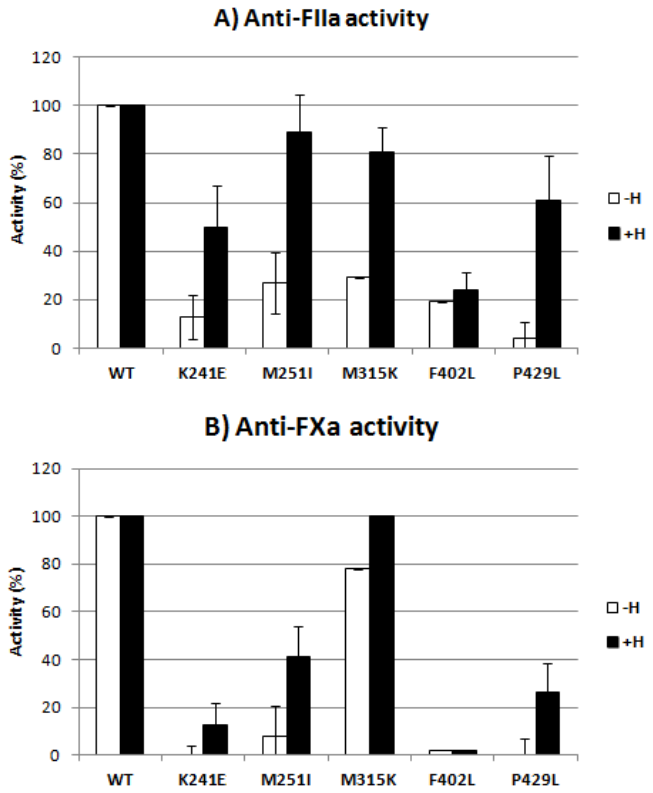


Figure 7. Inhibitory activity of antithrombin variants by chromogenic assays. Anti FIIa (A) and anti-FXa (B) activities of antithrombins secreted to the conditioned medium (V1 + V2) in absence (grey) or presence (black) of heparin (H)**.** Results are expressed as a percentage of the activity of the wild-type antithrombin (WT), which was used as reference (100%). Each bar represents the mean ± SD of two independent experiments performed in duplicate.

These results were confirmed with purified mutants evaluating the formation of thrombin-antithrombin complexes at different intervals of time (Figure 8A,B). The V1 of all mutants did not form covalent complexes with thrombin in presence or absence of heparin, even at long time of incubation (Figure 8 A). In contrast, V2 was able to form complexes with thrombin in presence of heparin (Figure 8 B), except for V2 from F402L. This mutation severely affected the reactivity of both glycoforms (Figure 8 A,B).

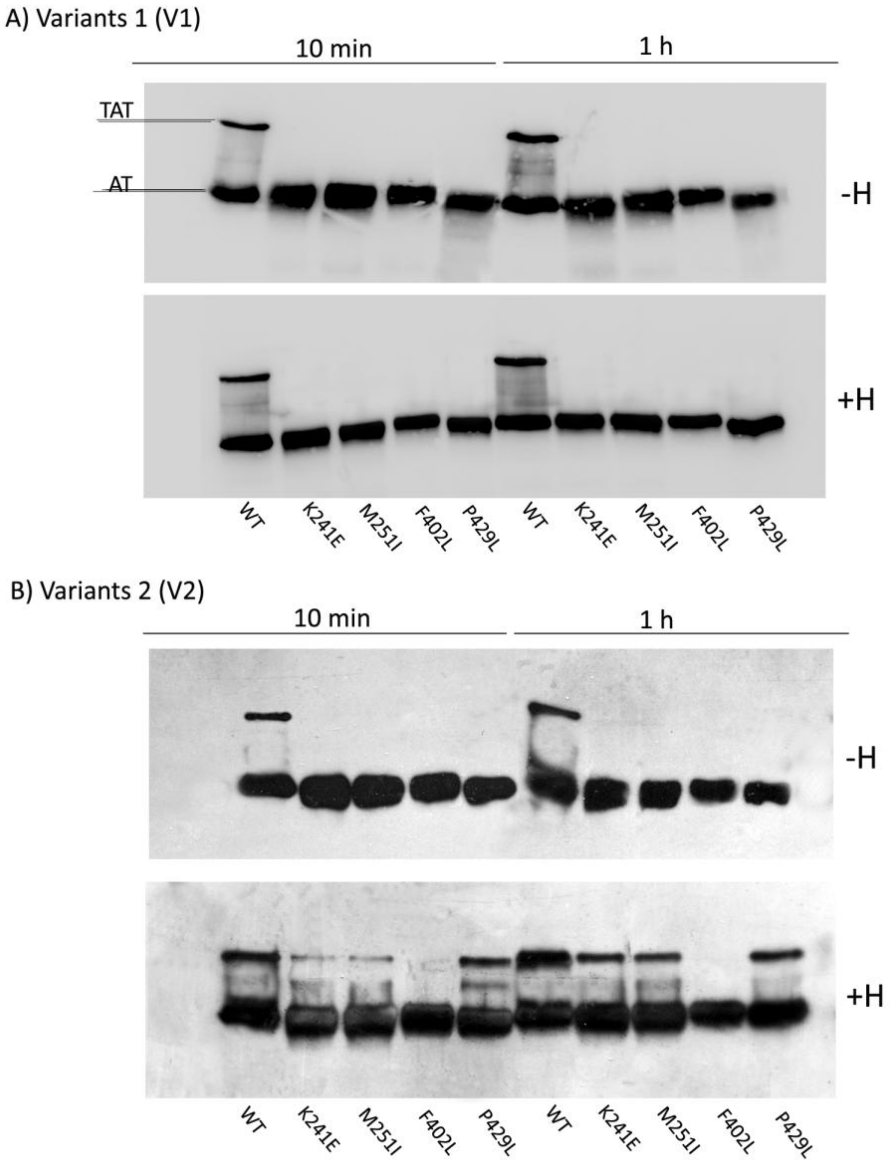


Figure 8. Formation of thrombin-antithrombin (TAT) complexes. The wild-type (WT) and V1 **(A)** and V2 **(B)** of each pleiotropic mutant in presence (+H) or absence of unfractionated heparin (-H) and identified by SDS-PAGE under reducing conditions and western blotting after 10 min or 1 h of incubation with thrombin.

We also evaluated the reactivity of pleiotropic glycoforms with HNE. As shown in figure 9, V1 from all the tested mutants, did not react with HNE as no cleaved antithrombin was detected in SDS-PAGE under non-reducing conditions, while this protease cleaved V2 of the different mutants studied.

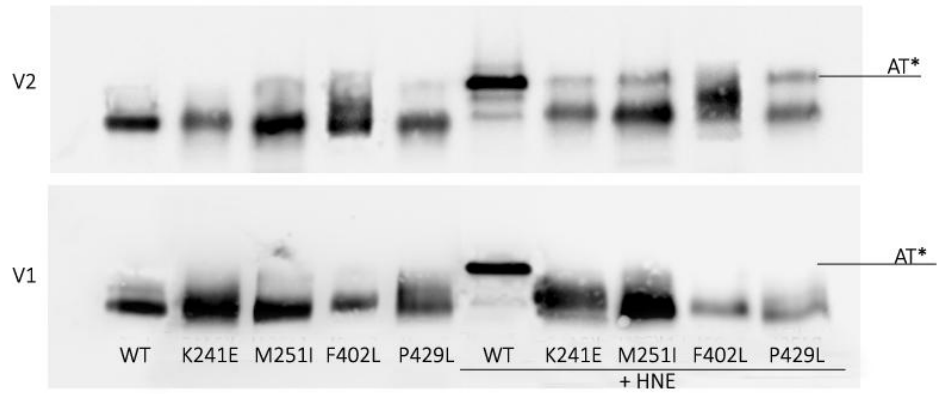


Figure 9. Electrophoretic separation by SDS-PAGE under non-reducing conditions and western blotting of variants 1 and 2 (V1 and V2) and wild type antithrombin (WT) after incubation with human neutrophil elastase (HNE) and unfractionated heparin during 30 min. The black line marks cleaved antithrombin (AT*).

3.6 Evaluation of the conformational stability of antithrombin variants

The SDS-PAGE analysis under non reducing conditions indicated that none of the variants presented disulfide-linked dimers (Figure 10 A), an oligomeric form that has been described associated with conformational mutations (Corral *et al.*, 2004). However, rules out the possibility that the absence of inhibitory activity, particularly of V1 might be due to non-inhibitory conformations, we evaluated the presence of hyperstable conformations by urea-PAGE. None of the mutants showed increased levels of hyperstable conformations, mainly latent form, in comparison with the wild-type molecule (Figure 10 B).

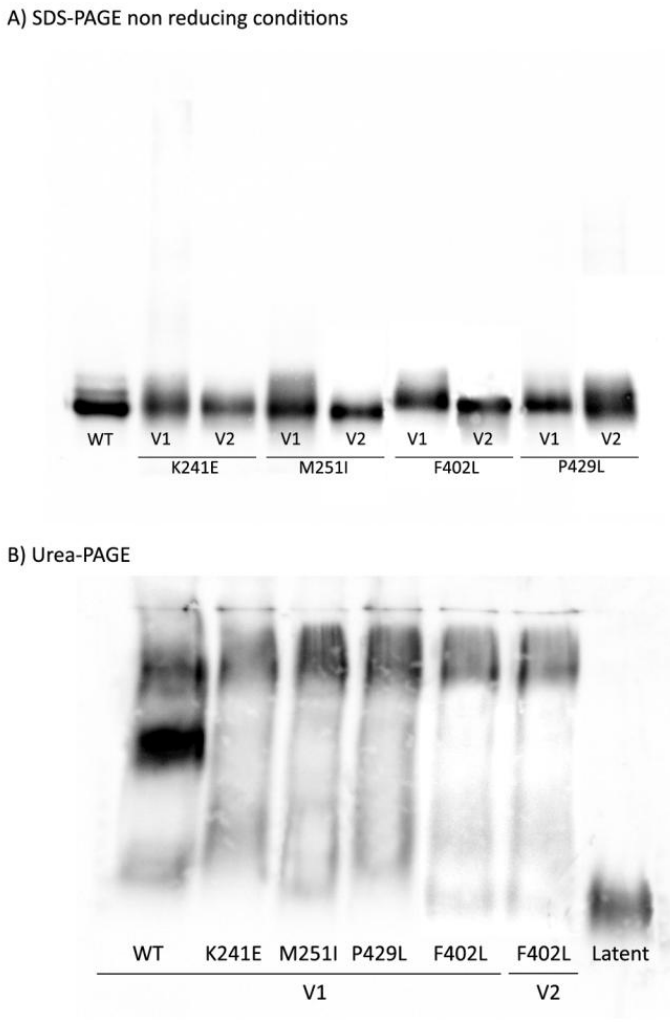


Figure 10. Evaluation of hyperstable conformations of purified variants of pleiotropic mutations. **A)** Electrophoretic separation by SDS-PAGE under non-reducing conditions and western blotting. **B)** Electrophoretic separations by urea-PAGE and western blotting. Latent antithrombin of plasma was used as control. Wild type antithrombin (WT) was used as control in both cases.

4. Discussion

The characterization of natural mutations identified in patients with antithrombin deficiency has helped to discover and characterize both functional domains of this key anticoagulant such as the heparin binding domain, as well as to define new mechanisms involved in the deficiency of this serine protease inhibitor (Blajchman *et al.*, 1992; Caspers *et al.*, 2012; Corral *et al.*, 2004; Chang *et al.*, 1986; Lane *et al.*, 1997; Martinez-Martinez *et al.*, 2010; Raja *et al.*, 2003). Moreover, these studies might also help to identify the clinical prognosis associated with each mutation (Martinez-Martinez *et al.*, 2012c; Martinez-Martinez *et al.*, 2010). This study aims to characterize a type of antithrombin deficiency scarcely studied so far, whose underlying mechanisms are not well known and with a wide range of clinical presentations: type IIc or pleiotropic. Thus, the analysis of these mutations has allowed the identification of a new functional region antithrombin that indirectly influences the heparin affinity and the anticoagulant capacity of this protein. Moreover, our data support the

relevance of an abnormal glycosylation associated with these mutations that might be involved in the clinical severity.

Different studies have suggested that glycosylation might play a role in both the physiology and pathology of this serpin (Garone *et al.*, 1996; Martinez-Martinez *et al.*, 2012c; Martinez-Martinez *et al.*, 2010; Olson *et al.*, 1997; Preston *et al.*, 2013). Interestingly, our group has recently demonstrated that a variable glycosylation modulates the effect of mutations affecting the binding of heparin (Martinez-Martinez *et al.*, 2012c). Thus, the absence of a carbohydrate confers to the β-glycoform an advantage for the interaction with the heparin as it lacks a steric hindrance for the interaction. An opposite situation, the fucosylation of the N-glycan at Asn155, explains the low heparin affinity of recombinant antithrombin forms expressed in baby hamster kidney cells, since this unusual glycan impairs the interaction with heparin (Garone *et al.*, 1996). Moreover, the fucosylation indirectly caused by the K241E mutation was also responsible for the low heparin affinity of this pleiotropic mutation (Martinez-Martinez *et al.*, 2010). In this study, we analyzed different pleiotropic mutations affecting the C-sheet in order to evaluate if there is a mechanism shared by this group of deficiencies. The analysis of these recombinant antithrombin mutants proved the presence of two glycoforms (V1 and V2). V1 exhibited less electrophoretic mobility in SDS gels under reducing condition than WT antithrombin and V2. These differences disappeared after N-glycosidase F treatment, as verified by MALDI-TOF analysis, indicating an abnormal composition of the carbohydrates associated with these mutations. Additionally, V1 showed a different electrophoretic mobility depending on the mutation. These results suggest that each mutation might cause a different glycan composition that could explain the heterogeneity of abnormal glycoforms associated with each pleiotropic mutation. Moreover, the direct identification of a fucose in the V1 of M251I mutant confirmed the alteration of the glycosylation induced by this mutation, which explains the reduced heparin affinity of this variant; and it supports that the differences between V1 and V2 are caused by different glycan content. It is important to point out that this is the first result that directly identifies a fucose in antithrombin with a missense mutation. However, further studies are required to establish the abnormal glycosylation of the V1 of the other pleiotropic mutations. Collectively, these results indicate that the C-sheet might be a region involved in the maturation of the N-glycosylation of antithrombin. However, the mechanism leading to such abnormal N-glycan maturation is unknown. Our first study suggested that the K241E mutation, by changing significantly the electrostatic potential of the molecule, might interfere the interactions with fucosyltransferases (Martinez-Martinez *et al.*, 2010), but this hypothesis cannot be extended

to the rest of mutations tested in this study, since all of them do not have an effect on the electrostatic potential. Moreover, these mutants have been generated in a β-context, lacking of glycosylation at Asn135, which points out that the reduced heparin affinity and reactivity of V1 observed in this study must be caused by the abnormal maturation of the N-glycan of the three other sites of glycosylation.

Interestingly, both V1 and V2, which share the same mutation and only differ in the N-glycan content, are not able to interact with thrombin in absence of heparin even with long times of incubation. These results support previous data suggesting that certain mutations on the C-sheet significantly interferes the serpin-protease interaction (Izaguirre *et al.*, 2006; Izaguirre *et al.*, 2007; Lane *et al.*, 1992). However, V1 does not present inhibitory activity even in presence of heparin, therefore, the mutation on the C-sheet and the alteration of the glycan content still impair the interaction with the target proteases. Moreover, the defective reactivity of V1 of these pleiotropic mutants also affects the interaction with HNE, explaining the absence of inhibitory activity and cleaved forms. In contrast, binding of heparin to V2 causes a correct activation of the molecule that seems to eliminate the deleterious consequences of the C-sheet mutation on the interaction with the proteases, allowing a full anticoagulant activity and the proteolytic activity of HNE, with the exception of F402L. Our data agree with those obtained by Lane and coworkers who suggested a conformational linkage between the reactive site and heparin binding regions of the molecule in s1C variants (Lane *et al.*, 1992).

In summary, mutations at the C-sheet have a common and relevant functional consequence: the mutation impairs the reactivity with the target proteases. However, this effect could be abolished in V2 by activation with heparin, since the glycosylation of these glycoforms seems to be similar to that of WT. The abnormal maturation of the N-glycan combined with the mutation present on V1 of pleiotropic mutations provoke that they have not reactivity and the presence of heparin does not restore the effect of the mutation.

The exception to this rule is F402L since the V2 apparently has normal glycosylation and heparin affinity, but it did not recover anticoagulant activity after activation with heparin. This feature might also explain the severe clinical phenotype reported for patients carrying this mutation (Emmerich *et al.*, 1994a; Lane *et al.*, 1992). As it has been demonstrated that T401 directly interacts with E37 of FXa (Johnson *et al.*, 2006b), we speculate that F402 might be relevant for the interaction with the target proteases and the mutation F402L might impair the interaction between these residues. Further studies are required to verify this hypothesis.

The potential role of a conformational effect of pleiotropic mutations on the functional consequences observed in this study was ruled out. It is well known that missense mutations

affecting the shutter or regions involved in the mobility of the RCL may affect the correct folding or stability of this protein leading to inactive conformations (Corral *et al.*, 2007; Mushunje *et al.*, 2004). Moreover, Bottomley and coworkers found that alterations in s1C of antitrypsin could cause the serpin to become unstable (Bottomley *et al.*, 2001). Additionally, it has been described that the lack of glycosylation in PAI-1 changes the transition to latency in this molecule (Bager *et al.*, 2013). However, our results show that none of the pleiotropic mutations evaluated in our study form disulfide-linked dimers and we do not detect increased levels of the latent form in these non-functional variants compared with the WT. Moreover, the V1 mutants are not in altered form (latent or polymerized) because fluorescence increase occurs when the serpins are incubated with heparin, this is diagnostic for a native-like conformation. Finally, the fact that V2, when activated by heparin, are able to have inhibitory activity and react with HNE, demonstrates the presence of an exposed RCL and a native conformation. Therefore, these data suggest that pleiotropic mutations do not cause a significant structural instability.

In conclusion, our study on recombinant pleiotropic mutants (K241E, M251I, F402L and P429L), identified in patients with antithrombin deficiency and thrombosis, reveals that the C-sheet seems to be relevant for the correct maturation of the N-glycans. The only exception is M315K mutant probably because the localization of this residue, close to the reactive center, might not affect the normal interaction with glycosyl transferases or glycosidases. Alternatively, these mutations might affect the folding and/or normal traffic of the protein from the endoplasmic reticulum to the *trans*-Golgi that might allow the interaction of antithrombin with enzymes involved in the N-glycosylation that normally hardly interact. Independently of the mechanism involved, the final result is an abnormal maturation of the N-glycans of antithrombin variants with mutations in the C-sheet that impair the heparin affinity and the reactivity. Finally, it is important to point out that our results agree with the clinical phenotype of patients carrying these mutations, explaining the different clinical severity observed in this group of antithrombin deficiencies.

IV. Stabilization of N-glycosylation on N135 of Antithrombin by an Aromatic Sequon

Aim 3

1. Introduction

The N-glycosylation is a post-translational modification that can be dispensable for the function of many glycoproteins but, in some cases, it may affect protein conformation or folding, and even the function and specificity of interaction (Ni Ainle et al., 2011; Preston et al., 2013). Moreover, glycosylation plays a key role in the transit system of proteins out of the endoplasmic reticulum, in their stability or half-life, in the sensitivity to proteases or others reactive substances, and in their immunogenicity (Hanson et al., 2009; Wormald et al., 1999). When this process does not occur properly or is inhibited, proteins may form aggregates and are degraded via proteasome (Helenius et al., 2004; Trombetta, 2003). The urgent need for glycan-defined glycoproteins in both detailed structure-function relationship studies and therapeutic applications has stimulated the development of various methods for manipulating protein glycosylation (Brooks et al., 2013; Elliott et al., 2003; Ni Ainle et al., 2011; Wang et al., 2012). In this framework, particular interest have two recent studies, in which it was evaluated the effect of an aromatic residue before the N-X-T, on the glycosylation efficiency and the stability of proteins (Culyba et al., 2011; Price et al., 2012; Price et al., 2011). The placement of a phenylalanine, two residues before the asparagine of N-glycosylation consensus sequence (aromatic sequon), situated in reverse turns increased the glycosylation efficacy and the global stability of different proteins due to the side chain interactions of phenylalanine with the hydrophobic α face of $GlcNAc_1$ on asparagine (Culyba et al., 2011; Price et al., 2012). This study also suggested that the presence of a threonine two residues after asparagine in the consensus sequence favors a more compact structure (Culyba et al., 2011). A more recent study from the same group also showed that the increased of the stability provoked by the interaction between the glycan and aromatic ring is more notable if this aromatic sequon is located in type I β-bulge turns other than reverse turns (Price *et al.*, 2011). However, as far as we do not know further study has evaluated the relevance of an aromatic sequon in other structural context or localizations.

The aim of this study was to evaluate the effect of an aromatic sequon (F-Y-N-X-T/S) on the glycosylation consensus sequence of N135 of antithrombin, which is inefficiently glycosylated in the wild-type sequence. To achieve this aim, we analyzed the glycosylation efficiency, inhibitory function, heparin affinity and structural stability of different antithrombin mutants. This is the first study evaluating the effect of this aromatic sequon in a different structural context, since the N135-K136-S137 glycosylation consensus sequence of

antithrombin lays in a loop between an α-helix and a β-strand, but not in a reverse turn, as has been described previously.

2. Material and Methods

2.1 Site directed mutagenesis and recombinant expression

We used the pCEP4-AT plasmid containing the cDNA sequence of human antithrombin and Human Embryonic Kidney cells expressing the Epstein Barr Nuclear Antigen 1 (HEK-EBNA) cells, generously provided by Prof. J. Huntington (CIMR, Cambridge, UK). This plasmid, commonly used to produce recombinant antithrombin in HEK-EBNA cells, had the S137A mutation to only produce β-antithrombin Firstly, we returned to the WT sequence (S137), and then, we generated the following mutations, K133F, S137T and K133F/S137T by site directed mutagenesis using the Stratagene Quik Change Site-Directed Mutagenesis kit (Agilent, Madrid, Spain) and the appropriate primers (Table S1). HEK-EBNA cells were grown to 80% confluence at 37 °C, 5% CO_2, in DMEM with GlutaMAX-I medium (Invitrogen, Barcelona, Spain) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Madrid, Spain). Transfection was performed by addition of plasmid (200 µg/mL) that it was preincubated for 30 minutes in serum-free OptiMEM culture medium with Lipofectamine LTX reagent (Invitrogen, Barcelona, Spain) according to the manufacturer's protocol. Sixteen hours post-transfection, cells were washed with PBS and exchanged into CD-CHO medium (Invitrogen, Barcelona, Spain) supplemented with 4 mM L-glutamine and 0.25 mg/mL geneticin (Invitrogen, Barcelona, Spain). Cells were grown to confluence and media was harvested every 42 h for 11 days.

Cells were extensively washed with sterile PBS and then lysated with 50 µl of lysis buffer (10 mM TrisHCl, 0.5 mM DTT, 0.035% SDS, 1mM EGTA, 50 mM sodium fluoride, 50 µM sodium orthovanadate, 5 mM benzamidine and 20 mM phenylmethylsulphonyl fluoride). The homogenate was centrifuged at 12000 rpm for 10 minutes at 4ºC and supernatants were kept stored at -80 °C. Separation of proteins was evaluated by SDS-PAGE under reducing conditions and immunoblotting. Monoclonal anti β-actin (Sigma-Aldrich, Madrid, Spain) was used to check sample loading.

2.2 Protein purification and electrophoretic evaluation

Recombinant proteins were purified by heparin affinity chromatography on 5 mL HiTrap Heparin columns (GE Healthcare, Barcelona, Spain), using an ÄKTA Purifier (GE Healthcare, Barcelona, Spain) in 50 mM Tris-HCl, pH 7.4 buffer where a gradient from 0 to 3 M NaCl was applied to elute the proteins. Next, fractions containing antithrombin

were further purified by anion exchange chromatography on 1 mL HiTrap Q columns (GE Healthcare, Barcelona, Spain) by applying a gradient from 0 to 1 M NaCl in 50 mM Tris-HCl pH 7.4. Those fractions containing pure antithrombin were finally desalted and stored at -70 ºC. Purity of proteins was evaluated by 8% SDS-PAGE by silver staining, as indicated elsewhere (Mushunje *et al.*, 2004).

2.3 MALDI-TOF-MS analysis

A solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 g/L) in acetonitrile (ACN)/water/trifluoroacetic acid (TFA) (50:50:0.1 by vol.) was chosen as matrix for protein analysis. Experiments were carried out on a 4800 Plus MALDI TOF/TOF™ Analyzer (ABSciex), equipped diode-pumped, solid-state laser. Recorded data were processed with Data Explorer™ Software (Applied Biosystems, California, USA).

2.4 Differential scanning calorimetry (DSC)

Data collection and analysis were performed using Microcal MC-2 scanning microcalorimeter (Microcal Inc., Northamptom, MA) and Microcal Origin 5.0 (Microcal Software Inc., Northamptom, MA), respectively. The typical scan rates were 60 ºC/h. The denaturation heat capacity change (ΔC_p) was determined as the difference between the heat capacity of the native and the denatured forms of each protein sample extrapolated to the melting temperature (*Tm*) (Torrecillas *et al.*, 2004; Wen *et al.*, 2012). The proteins were prepared in the appropriate buffer (50 mM Tris HCl at pH 7.4) and the final concentration was 0.5 mg/mL.

2.5 Stopped flow

The kinetics of heparin binding were continuously monitored from the increase in protein fluorescence in an spectrometer π*-180 (Applied Photophysics) connected to a stopped-flow kinetic sample handling unit, as described previously (Olson *et al.*, 1981b) with a range of concentration of pentasaccharide from 4 nM to 30 µM (always in at least 5-fold molar excess of pentasaccharide to ensure under pseudo-first order conditions). For reactions at inhibitor concentrations less than 1µM, two reaction curves were typically averaged for each rate constant determination. All measures were conducted at 25 ºC in pH 7.4 buffers consisting of 20 mM sodium phosphate; 0.1 mM EDTA; 0.1% polyethylene glycol 8000; and 0.1 M NaCl to achieve ionic strengths of 0.15.

2.6 Determination of dissociation equilibrium constant (K_D) and inhibitory function

Equilibrium dissociation constant for the antithrombin-heparin interaction were determined by intrinsic fluorescence analysis performed essentially as described previously (Langdown *et al.*, 2009; Olson *et al.*, 1981a). Briefly, changes in intrinsic fluorescence of antithrombin (25

nM) upon titration of unfractionated heparin (Rovi, Madrid, Spain) were monitored at 340 nm on a Hitachi F4500 spectrofluorometer, with excitation at 280 nm and using bandwidths of 3.5 nm for both excitation and emission. All titrations were carried out at room temperature under physiological ionic strength (I = 0.15) in 20 mM NaPO4, 100 mM NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4. Fluorescence emission intensity was taken as the average of 100 measurements recorded at one second intervals for each addition of heparin. Data were fitted as previously described (Langdown *et al.*, 2009; Olson *et al.*, 1981a)**.**

Anti-FXa activity was evaluated using 400 ng of total purified protein and the chromogenic method, as described elsewhere (Corral *et al.*, 2004).

3. Results

3.1 Evaluation of the glycosylation efficiency

First all variants rendered similar amounts of antithrombin to the conditioned medium 24 h after transfection and supporting that none of the mutants significantly impaired the folding or intracellular traffic (Figure 1). Then, we compared the efficiency of glycosylation at Ans135 of the three different mutations affecting the wild-type (WT) glycosylation consensus sequence (**N135**-K136-**S137**) by evaluating the electrophoretic mobility of the secreted protein to the medium. As shown in figure 1, the transfection with the WT plasmid resulted in a similar production of α and β-glycoforms. Moreover, the mutation of S137 residue to Ala (S137A) only produced β-antithrombin (Picard *et al.*, 1995) (Figure 1). In contrast, the mutation of this residue to threonine (S137T) turned into the production of only α-antithrombin (Figure 1), as previously described (Picard *et al.*, 1995). Interestingly, the inclusion of an aromatic residue by the K133F mutation, two residues before N135 in a WT context (**K133**-A134-**N135**-K136-**S137**), fully increased the glycosylation efficiency because it only yielded α-antithrombin (Figure 1 & 2). Finally, the combination of both mutations, K133F and S137T, also exclusively produced α-antithrombin (Figure 1 & 2). These results were confirmed by mass analysis of purified proteins (MALDI-TOF MS) (Figure 2).

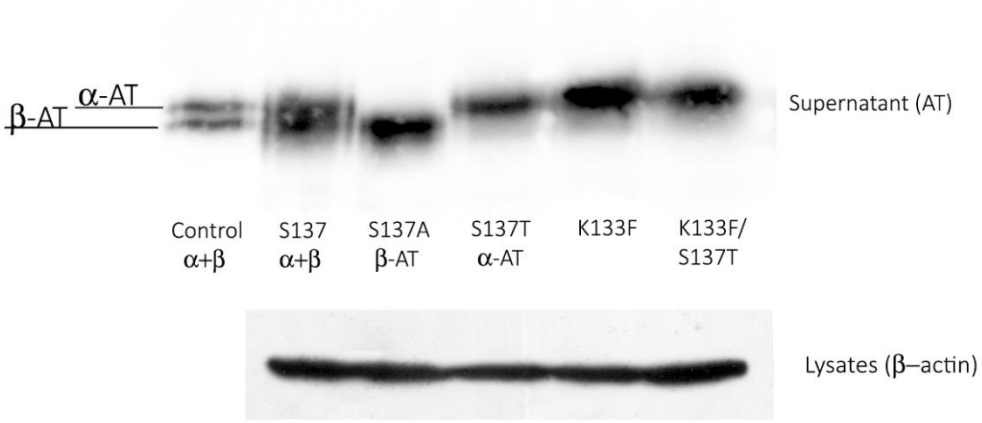


Figure 1. Expression of antithrombin variants. SDS-PAGE under reducing conditions and western blot of recombinant antithrombin variants from intracellular lysates or conditioned medium of HEK-EBNA cells 24 h after transfection. As load control in cellular lysates we showed the expression of β-actin. As control of electrophoretic mobility we used a mixture of α and β-antithrombin purified from plasma of healthy subjects.

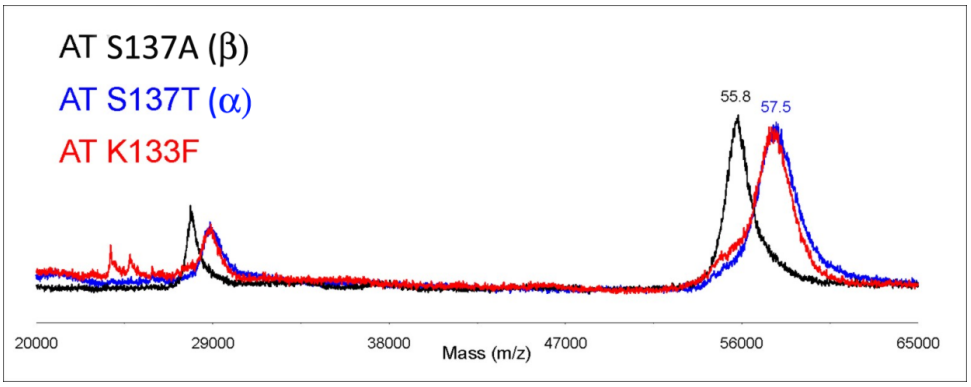


Figure 2. MALDI-TOF mass spectra of purified antithrombin variants.

3.2 Thermal stability of purified antithrombin variants

After purification, proteins were concentrated and used for DSC analysis. Our results showed similar melting temperatures for all proteins: 51.48 ± 1.54 ºC. Data obtained from α and β-glycoforms of antithrombin purified from plasma also had similar melting temperatures (data not shown).

3.3 Kinetics of Heparin Binding

The pentasaccharide binds to antithrombin in an initial rapid equilibrium step characterized by a dissociation constant, K_1, and then induces a conformational changes in the protein with forward and reverse rate constants, k_{+2} and k_{-2} (Scheme 1).

Scheme 1.

$$AT + H \underset{}{\overset{K_1}{\rightleftharpoons}} AT^{\bullet}H \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AT^{*\bullet}H$$

Scheme 1: Initial rapid equilibrium, $\mathbf{K_1}$, between antithrombin, **AT**, and pentasaccharide, **H**, leads to complex, $\mathbf{AT^{\bullet}H}$, followed by rapid conformational change via $\mathbf{k_2}$ to a high heparin affinity, highly fluorescence complex, $\mathbf{AT^{*\bullet}H}$.

The pentasaccharide binding of antithrombin variants were monitored by the increase of intrinsic fluorescence of protein under pseudo-first order conditions. The presence of aromatic residue (K133F or K133/S137T mutants) dramatically affected the conformations change step by pentasaccharide, as K_1; k_{+2} and k_{-2} were not measurable in the same conditions and concentrations than that used for control antithrombin (Table 1). The aromatic ring of phenylalanine is not by itself the responsible of this effect, since the K133F/S137A mutant, with three N-glycans (data not shown), only moderately impaired the binding affinity of pentasaccharide compared to β-antithrombin (S137A) (Table 1).

Table 1. Stopped-flow. The antithrombin-pentasaccharide binding data were measured as described in Materials and Methods section.

Variants	K_1 (μM)	k_2 (s^{-1})	k_{-2} (s^{-1})
β-AT (S137A)	4.9 ± 0.8	374.0 ± 5.4	0.6 ± 0.2
K133F/S137A	26.5 ± 0.5	461.0 ± 1.8	0.7 ± 0.4
K133F	ND	ND	ND
K133F/S137T	ND	ND	ND

3.4 Heparin affinity and functionality

The effect of these mutations was also evaluated on two functional features of antithrombin: heparin affinity and inhibition of FXa.

We analyzed the influence of the aromatic sequon on the heparin affinity determined by the changes of the intrinsic fluorescence caused by the titration with unfractionated heparin. As shown in table 2, the β-glycoform (S137A) had higher heparin affinity than the α-glycoform (S137T), as previously reported (Martinez-Martinez *et al.*, 2012c). However, the K133F mutant had dissociation constant (K_D) slightly higher than control variant (S137T). In addition, double mutant (K133F/S137T) had a four-fold lower heparin affinity (Table 2).

Variants	K_D (nM)	(ΔF max/Fo) x100
β-AT (S137A)	37.6 ± 3.1	41.0
α-AT (S137T)	48.9 ± 2.5	43.4
K133F	81.4 ± 3.6	44.1
K133F/S137T	195.4 ± 4.0	44.5

Table 2. Interaction of different antithrombin variants with heparin. The equilibrium dissociation constant (K_D) and maximal fluorescence (($\Delta F\ max/F_0)x100$) increase for the binding of heparin on antithrombin variants were measured by global fitting of fluorescence titrations with unfractionated heparin as described in Materials and Methods section.

Functional analysis by using chromogenic assays verified the consequences of these impaired heparin affinity induced by the aromatic sequon. Thus, the β-glycoform generated by mutation at residue 137 (S137A) had similar inhibitory activity (anti-FXa) than the α-glycoform used as reference (S137T) (Figure 3). In contrast, the α-glycoform generated by the aromatic sequon (K133F) had a weak reduction of its inhibitory activity (80.3%), which was more severe in the double mutant (K133F/S137T) (55.2%) (Figure 3).

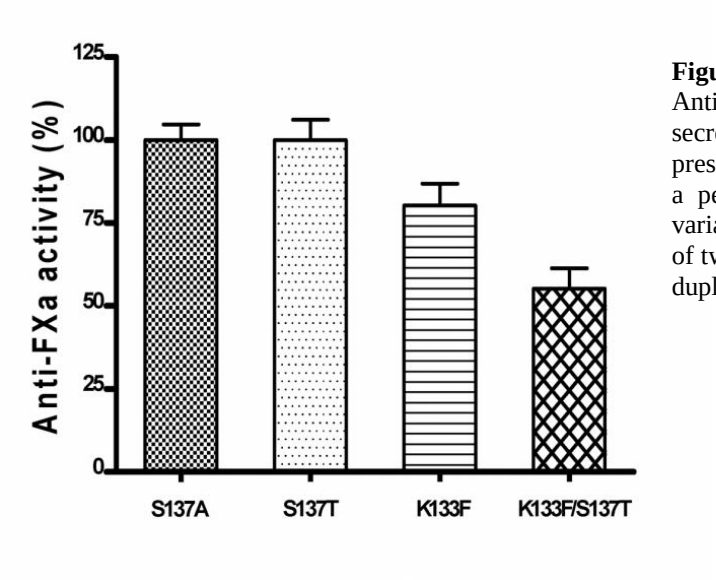


Figure 3. Function of antithrombin variants. Anti-FXa activity of antithrombin proteins secreted to the conditioned medium in presence of heparin**.** Results are expressed as a percentage of the activity of the S137T variant. Each bar represents the mean ± SD of two independent experiments performed in duplicate.

4. Discussion

Protein glycosylation is a complex post-translational modification crucial for proteins, since it determines its functional specificity, clearance and stability. These features together with the increasing use of recombinant proteins, particularly for therapeutic applications, highlight the relevance of emerging glycoengineering technologies aiming to reduce heterogeneity of glycoforms, to improve protein stability or increase the half life (Brooks *et al.*, 2013; Elliott *et al.*, 2003; Hanson *et al.*, 2009; Liu *et al.*, 2009; Price *et al.*, 2012). The final goal is to achieve the desired therapeutic efficacy of the recombinant molecule. In this framework, two recent studies from the same group have showed that the N-linked glycans have an influence on the folding and enhance the stability of proteins (Culyba *et al.*, 2011; Price *et al.*, 2012; Price *et al.*, 2011). These works show that the incorporation of an aromatic sequon (Phe–Y–Asn–X–Thr, where Y can likely be any amino acid, and X is any amino acid but proline) is an interesting strategy for enhancing an efficient glycosylation and for increasing the stability of many proteins that harbor reverse β-turns (Culyba *et al.*, 2011; Price *et al.*, 2012; Price *et al.*, 2011). Our study demonstrated that this strategy may also be useful to improve the glycosylation efficiency in other structural contexts. The WT antithrombin, with N135-K136-S137 glycosylation consensus sequence, is inefficiently glycosylated, as the 50% of molecules are glycosylated at this position. Although this sequence is not located in the reverse turn (none of the glycosylation sites of antithrombin is placed in a reverse β-turn), but in a loop between helix D and strand 2A (Figure 4), the presence of an aromatic residue (Phe) two residues before the asparagine residue (*i* and *i+2*, respectively, using the nomenclature of Culyba et al., 2011) allows a complete N-glycosylation of this site, similarly to that caused by the S137T mutation.

The aromatic sequon in reverse β-turns also increased stability of different proteins (rat CD2 adhesion domain, human muscle acyl phosphatase and WW domain of the human protein Pin1) (Culyba *et al.*, 2011). This effect was explained by the hydrophobic interactions between the α-face of GlcNAc1 and the aromatic ring of phenylalanine at (*i*) position together with the C-H⁄π interaction (Culyba *et al.*, 2011; Price *et al.*, 2011). Thus, we evaluated the effect of the aromatic sequon on the structural stability of this sensitive serpin. The DSC study of purified variants confirmed that the presence of a phenylalanine at (*i*) position, despite improving the efficiency of N-glycosylation on consensus sequence of N135, does not increase the global stability of this protein. It is possible that the position of the aromatic sequon in a loop might explain this effect, but we think that the stability caused by the aromatic sequon may never compensate the instability of the stressed native conformation of antithrombin, i.e. a metastable conformation required for an efficient inhibitory mechanism (Carrell *et al.*, 1985; Huntington *et al.*, 2000), that also provides antithrombin vulnerable to environmental or genetic factors, like other serpins (Carrell *et al.*, 2003; Gettins, 2002; Hernandez-Espinosa *et al.*, 2007).

However, the aromatic sequon might still have some structural consequences, since this anticoagulant protein requires the activation induced by heparin to achieve a full anticoagulant activity (activated conformation) (Huntington, 2003; Olson *et al.*, 2010) and this glycosylation site is located close to the HBS. In fact, we considered that the aromatic sequon might stabilize the native conformation, hindering the conformational changes triggered by heparin activation (Figure 4) (Belzar *et al.*, 2002). Actually, the results obtained by stopped flow assay seem to support this idea. Moreover, the impaired heparin affinity of mutants with aromatic sequon has functional consequences, and these variants have reduced anticoagulant activity.

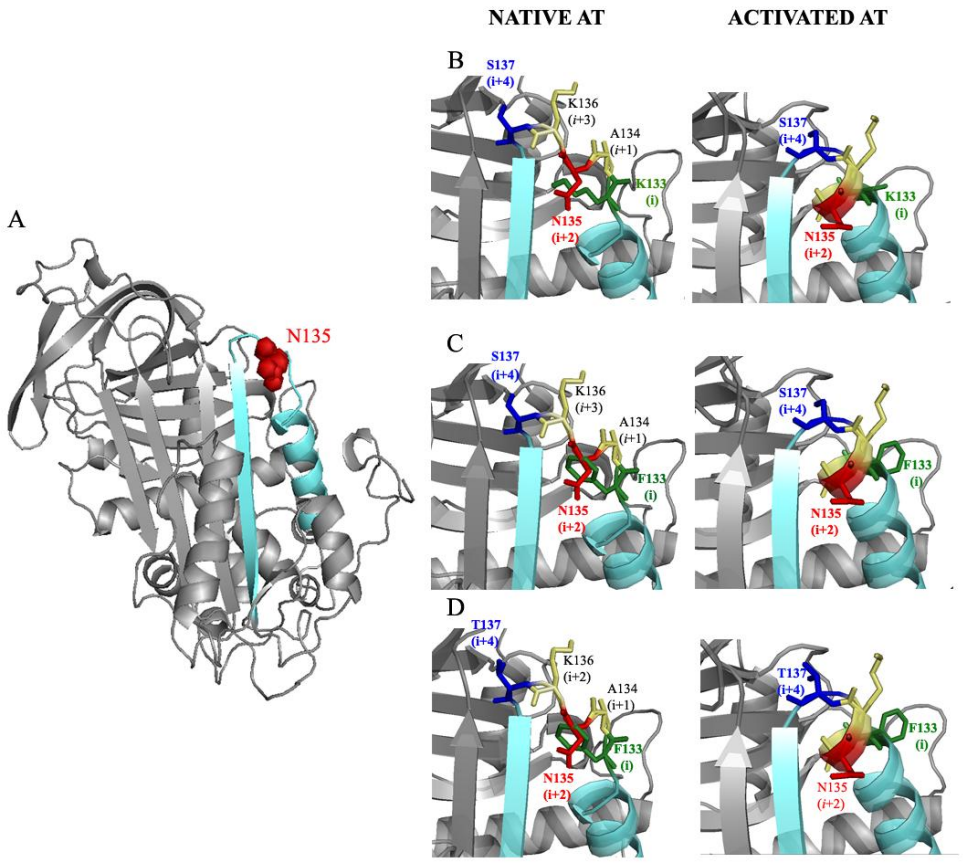


Figure 4. Ribbon diagram of antithrombin. **A)** Structural representation of the localization of N135 (red spheres) in native antithrombin (PDB code 1T1F). Strand 2A and helix D are displayed in cyan. **B)** Stick representation of the WT glycosylation consensus sequence (K133/S137 or (*i*) and (*i*+4) position); **C)** K133F mutant and **D)** double mutant (K133F/S137T) on native and activated antithrombin (AT) (PDB code 1T1F and 2GD4, respectively). All structures were rendered in Pymol (www.pymol.org).

In conclusion, the presence of an aromatic sequon generated by molecular engineering on the defective glycosylation consensus sequence of human antithrombin allows a fully efficient N-glycosylation process, leading to a homogeneous production of recombinant α-antithrombin, despite this sequence is not located in a reverse β-turn. This modification does not interfere with the global structural stability of antithrombin, but the new interactions caused by phenylalanine stabilize the native conformation of this protein, hindering the activation induced by heparin and its function. Further studies are required to verify whether or not this strategy might be useful to improve glycosylation efficiency and stabilization of proteins in other tertiary structures, apart from reverse turns, which may be useful in glycoengineery (Elliott *et al.*, 2003; Liu *et al.*, 2009; Sinclair *et al.*, 2005).

V. Generation of new N-glycosylation sites on the reactive center loop of antithrombin causes type IIa deficiency and determines specificity to proteases

Aim 3

1. Introduction

Antithrombin is an N-glycoprotein with four potential N-glycosylation sites. Modifications of its glycosylation have been associated with pathological conditions. Thus, the N135T mutation, identified in a family presenting borderline deficiency, removes this glycosylation site generating a variant protein

similar to β-antithrombin (Bayston *et al.,* 1999). On the other hand, abnormal N-glycosylation of antithrombin associated with antithrombin deficiency and risk of thrombosis is present in patients with congenital disorders of glycosylation (de la Morena-Barrio *et al.*, 2012b). We also identified a mutation in the *SERPINC1* (K241E) that by affecting the maturation of the N-glycan reduced the heparin affinity (Martinez-Martinez *et al.,* 2010). Recent data obtained in a recombinant model confirmed that other pleiotropic mutations caused the same effect (Aguila *et al.*, 2014). Indeed, core fucosylation of the N-glycan at N155, which is produced when antithrombin is expressed in other cells (CHO or BHK), reduced heparin affinity and impaired anticoagulant activity (Bjork *et al.,* 1992; Garone *et al.,* 1996). *SERPINC1* mutations that create a new N-glycosylation site have been identified in patients with antithrombin deficiency. The I7N missense mutation was identified in a proband presenting with type IIb antithrombin deficiency and the variant, with low heparin affinity, had an additional N-glycan (Brennan *et al.*, 1988). The second example is the missense mutation S82N, identified in a patient that presented a type I antithrombin deficiency. Recombinant expression of the N82 variant in COS-7 cells resulted in intracellular accumulation of the variant, that had an additional N-glycan, and no secretion of the protein was detected in the culture supernatant (Fitches *et al.,* 2001). The objective of this study was to evaluate whether new N-glycosylation sites in the RCL might cause type IIa deficiency, since they have never been described in patients with antithrombin deficiency, and to study the consequences of an N-glycan at such a functional region of this serpin.

2. Material and Methods

2.1 Recombinant expression of wild-type and mutant antithrombins

Site-directed mutagenesis (Stratagene QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) on the pCEP4/AT-S137A plasmid that produces β-glycoform of human antithrombin (with 3 glycans), was used to generate selected mutations that create new N-glycosylation sequences in different positions of

the RCL. As a control, we also used the pCEP4/AT plasmid with the S137T mutation that only generates α-antithrombin with 4 glycans.

Human Embryonic Kidney cells expressing the Epstein Barr Nuclear Antigen 1 (HEK-EBNA) were grown to 60% confluence at 37 °C, 5% CO_2, in DMEM with GlutaMAX-I medium (Invitrogen, Barcelona, Spain) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Madrid, Spain). We transfected 200 µg/mL of wild-type (WT) or mutant plasmids for 30 minutes in OptiMEM with lipofectamine LTX (Invitrogen, Barcelona, Spain), following the recommendations of the manufacturer. After 24 hours, the cells were washed with PBS and exchanged into CD-CHO medium (Invitrogen, Barcelona, Spain) supplemented with 4 mM L-glutamine and 0.25 mg/mL Geneticin (Invitrogen, Barcelona, Spain).

2.2 Evaluation of antithrombin variants by electrophoretic analysis in the conditioned medium and intracellular lysate

Transfected cells were grown in CD-CHO medium at 37 ºC for 24 hours and then the conditioned media was harvested and stored at -70 ºC. Cells were extensively washed with sterile PBS and then lysated with 50 µl of lysis buffer (10 mM TrisHCl, 0.5 mM DTT, 0.035% SDS, 1 mM EGTA, 50 mM sodium fluoride, 50 µM sodium orthovanadate, 5 mM benzamidine and 20 mM phenylmethylsulphonyl fluoride). The lysate was stored at -70 ºC.

Intracellular and secreted antithrombins were evaluated by Western blotting in 8% SDS-PAGE under reducing conditions. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich), followed by donkey anti-rabbit IgG–horseradish peroxidase conjugate (GE Healthcare, Barcelona, Spain), with detection *via* an ECL kit (Amersham Biosciences, Piscataway, NJ). Monoclonal anti β-actin (Sigma-Aldrich, Madrid, Spain) was used to check sample loading.

2.3 N-glycosidase F treatment

Conditioned medium was treated with N-glycosidase F (Roche Diagnostics GmbH, Mannheim, Germany) following conditions described elsewhere (Martinez-Martinez *et al.*, 2010). Briefly, a previous denaturing step (5 min at 100 ºC in 150 mM sodium phosphate buffer, pH 7.4 and 10% NP-40), samples were chilled on ice and digested with 0.6 U N-glycosidase F by incubation at 37 ºC over night. Samples were resolved by 10% SDS-PAGE under reducing conditions as described above.

2.4 Complexes formation with target proteases

Conditioned medium of cells transfected with wild-type and variants of antithrombin was incubated with FXa (8 µ) or FIIa (19 µM) for 10 min at 37 ºC. The reaction was carried out with and without a previous incubation with 6.6 µM of unfractionated heparin (Rovi, Madrid, Spain), for 10 minutes. The final reactions were loaded in SDS gels under reducing and non reducing conditions, and antithrombin (native monomer, cleaved or forming covalent complexes with the target proteases) detected by Western blot, as described above.

2.5 Assay conditions

Antithrombin-protease reaction stoichiometries and kinetics with thrombin and factor Xa were done with purified variants of antithrombin at 25 °C in 20 mM sodium phosphate, 0.1 M NaCl, 0.1 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.4 buffer . The ionic strength of buffer was 0.15.

2.6 Stoichiometry of antithrombin-protease reactions

The stoichiometries for the reactions of recombinant antithrombins with thrombin and factor Xa were determined as described previously (Olson *et al.*, 1993). Briefly, to fixed concentrations of 100 nM protease was added increasing concentrations of inhibitor to give molar ratios of inhibitor to protease of up to 2.8 in a final volume of 100 µl. Reactions were done both in the absence and presence of the full-length heparin or pentasaccharide, which was fixed at a concentration equimolar with the protease. After incubating for times sufficient to complete the reaction (95%), 5 µl of the reaction mixture was added to 1 ml of substrate (100 µM S-2238 for thrombin or 100 µM Spectrozyme FXa for factor Xa), and the residual enzymatic activity was measured from the initial linear rate of change of absorbance at 405 nm. The decrease in protease activity with increasing molar ratio of inhibitor/protease was fit by linear regression to obtain the stoichiometry from the abscissa intercept.

2.7 Kinetics of Antithrombin-Protease Reactions

Association rate constants for reactions of recombinant antithrombins with proteases in the absence or presence of pentasaccharide or full-length heparin were measured under pseudo-first order conditions by using at least a 10-fold molar excess of inhibitor over

protease as in previous studies (Bedsted *et al.*, 2003; Izaguirre *et al.*, 2003). For reactions with all proteases in the absence of heparin and those reactions with proteases in the presence of pentasaccharide whose rate constants were 10^4 $M^{-1}s^{-1}$ or less (or in some cases as high as 10^5 $M^{-1}s^{-1}$), full reaction time courses of the loss of enzyme activity were obtained and fit by a single exponential function with a zero activity end point.

Pentasaccharide or heparin was present in these full time course experiments at levels that saturated antithrombin. Assays of residual enzyme activity were done by removing aliquots of the reaction at different times, diluting into appropriate chromogenic substrates, and measuring the initial rate of substrate hydrolysis as in previous studies (Yang *et al.*, 2002). Association rate constants for free antithrombin or antithrombin-pentasaccharide/heparin complex reactions were obtained from fitted exponential rate constants by dividing by the concentration of antithrombin. For all other reactions in the presence of pentasaccharide or full-length heparins, reactions were done for a fixed time as a function of the concentration of heparin, and the loss in enzyme activity was fit by a single exponential function with heparin concentration instead of time as the independent variable (Izaguirre *et al.*, 2003). The association rate constant for the reaction of the antithrombin-heparin complex was obtained from the fitted exponential rate constant by dividing by the fixed reaction time and by a factor that corrected for the fraction of heparin that was bound by antithrombin.

3. Results

3.1 Identification of natural mutations potentially creating new N-glycosylation sites and selection of mutations for recombinant analysis

The search in available *SERPINC1* mutation databases as well as in large series of patients with antithrombin deficiency (Caspers *et al.*, 2012; Luxembourg *et al.*, 2011) (HGMD: http://www.hgmd.org/); and our own serie of 142 patients with congenital antithrombin deficiency, identified four different mutations able to create a new N-glycosylation sequences (Table 1).

Table 1. Natural missense mutations identified in patients with antithrombin deficiency creating new N-glycosylation sequences. (* The 32 amino acid signal peptide has been removed. ** Amino acid +1 is the methionine encoded by the ATG initiation codon).

Mutation*	Mutation **	Location	AT deficiency	Reference
Ile7Asn	p.Ile39Asn	N-terminal	Type IIb	Brennan *et al.*, FEBS Letter, 1988
Ser82Asn	p.Ser204Asn	hB (shutter)	Type I	Fitches *et al.*, Thromb Haemost, 2001 and our database
Ile219Thr	p.Ile251Thr	s3A	Type I	Picard *et al.*, Br J Haematol. 2000
Pro407Thr	p.Pro439Thr	Loop s1C-s4B	Type IIc	Lane *et al.*, J Clin Invest. 1992

According to these data, mutations generating a new N-glycosylation site may be responsible for all types of antithrombin deficiency, except for type IIa. So, in this study we evaluated the effect of two mutations generating sequences potentially glycosylated at the RCL: G392N, affecting P2, close to the crucial R393 residue (P1), and A384N (P10), proximal to the hinge region. Both residues have been described mutated in patients with type IIa deficiency, but the mutations identified in these patients did not create the consensus sequence of N-glycosylation (Blajchman *et al.*, 1992; Perry *et al.*, 1991).

3.2 Secretion and intracellular retention of antithrombin mutants

Evaluation of secreted variants to the conditioned medium by electrophoretic analysis in 8% SDS-PAGE under reducing conditions revealed that both RCL mutations were efficiently secreted, with levels in the conditioned medium similar to that observed for control antithrombins (α- and β-antithrombin) (Figure 1). The new N-glycosylation sites were efficiently glycosylated according to the reduced electrophoretic mobility, similar to α-antithrombin, which corresponded to the four glycans form (Figure 1). N-glycosidase F treatment of the conditioned medium equalized the size of antithrombin in all cases, demonstrating that N-glycosylation was the only element responsible for the differential size of these molecules (Figure 1A).

Moreover, the main intracellular antithrombin form for both mutants also had four N-glycans and the levels were similar to that observed in control (α and β-antithrombin) (Figure 1B).

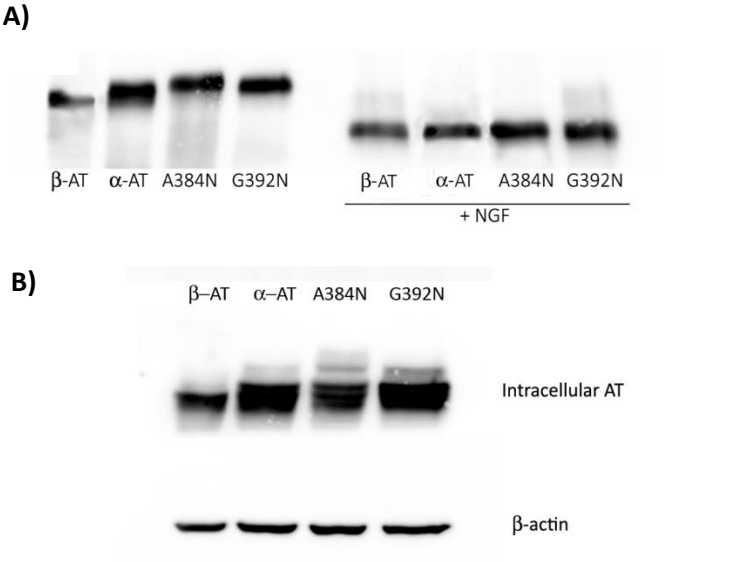


Figure 1. Electrophoretic separation by SDS-PAGE under reducing condition and western blotting of recombinant antithrombin (AT). A) Secreted of antithrombin variants to the conditioned medium of HEK-EBNA cells with N-glycosidase-F (+NGF) and without NGF treatment. B) Intracellular antithrombin variants from the same cells. As a loading control, the western blotting of β-actin is also showen.

3.3 Analysis of complexes formation with target of proteases

Both RCL variants evaluated in this study did not form complexes with FIIa in absence of heparin (Figure 2A). However, the reactivity with target proteases was different accordingly to the position of the N-glycan in the RCL and the activation by heparin (Figure 2). Thus, the glycan located at N384 did not block the interaction with the target proteases, but fully inhibited itsthe loop insertion. Indeed, the N384 variant behaved as a substrate, and all protein that reacted with both proteases was cleaved in presence and absence of heparin (Figure 2A & B). In contrast, the glycan in the N392 hampered the interaction with thrombin in absence of heparin as neither thrombin-antithrombin complexes nor cleaved antithrombin were detected (Figure 2A). Interestingly, activation by heparin of the Asn392 variant unlocked the capacity to interact with this protease, and even more, allowed the anticoagulant activity, as covalent complexes were observed (Figure 2A). Much more interest were the results obtained when the interaction of the Asn392 variant with FXa was evaluated. The presence of an N-glycan in P2 did not impair the inhibition of FXa neither in native nor activated state (Figure 2B).

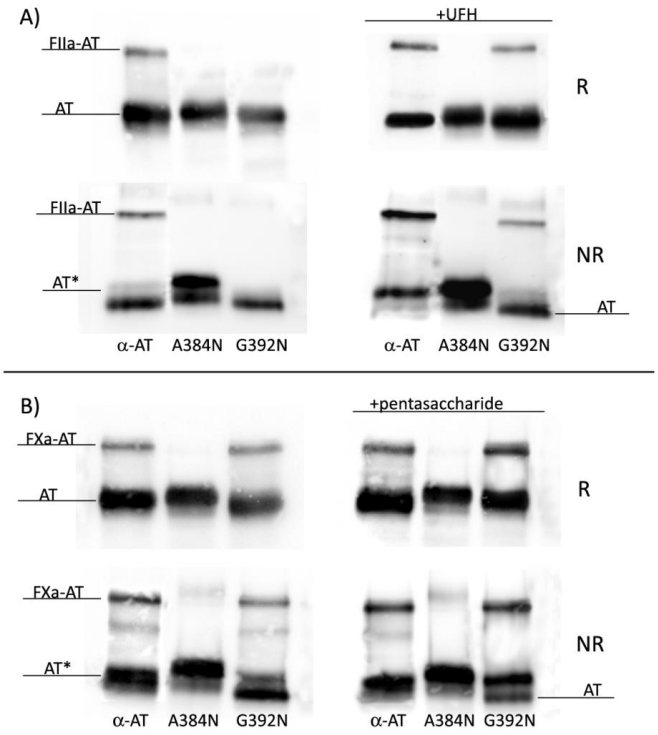


Figure 2. Formation of covalent complexes between antithrombin and its target proteases. Wild type (WT, α-antithrombin) and antithrombin mutants were incubated **(A)** with thrombin (FIIa) or **(B)** Factor Xa (FXa), in absence and presence of unfractionated heparin (+UHF) or pentasaccharide (+pentasaccharide), respectively. Conditioned medium was used for these experiments and antithrombin was detected by Western blot after SDS-PAGE under reducing (R) or non-reducing conditions (NR). (Antithrombin: AT, cleaved antithrombin: AT*, Thrombin-antithrombin complex: FIIa-AT, Factor Xa-antithrormbin complex: FXa-AT).

3.4 Reactivity of β-antithrombin and G392N mutant

The G392N variant did not react with thrombin in absence of full length heparin, despite to give molar ratios of inhibitor to protease of up to 10 (Table 1). Activation by heparin overcame the effect of the mutation, although the rates of thrombin inhibition were 10,000-fold lower than with control antithrombin (Table 2). Surprisingly, this mutant showed basal and activated rates with FXa inhibition similar to the β-antithrombin, but the stoichiometry in absence of pentasaccharide was higher (4-fold) (Table 2).

Table 2. Association rate constants (Kapp x SI) and inhibition stoichiometries (SI) with factor IIa (FIIa) and factor Xa (FXa) for control antithrombin (β-antithrombin) and G392N mutant. Values were measured as described in Material and Methods. H5, pentasaccharide; UFH, unfractionated heparin.

		FIIa		
		Kapp ($M^{-1} s^{-1}$)	**SI**	**Kapp x SI**
Control	-	$(5.8 \pm 0.1) \times 10^3$	1.2 ± 0.1	$(7.0 \pm 0.2) \times 10^3$
	+UFH	$(1.3 \pm 0.1) \times 10^7$	1.7 ± 0.1	$(2.2 \pm 0.6) \times 10^7$
G392N	-	Not reactivity		
	+UFH	$(7.6 \pm 0.1) \times 10^3$	2.6 ± 0.1	$(2.0 \pm 0.1) \times 10^4$
		FXa		
		Kapp ($M^{-1} s^{-1}$)	**SI**	**Kapp x SI**
Control	-	$(4.3 \pm 0.3) \times 10^3$	1.1 ± 0.1	$(4.7 \pm 0.5) \times 10^3$
	+H5	$(7.3 \pm 0.2) \times 10^5$	1.3 ± 0.1	$(9.5 \pm 0.6) \times 10^5$
G392N	-	$(7.1 \pm 0.2) \times 10^2$	4.9 ± 0.1	$(3.5 \pm 0.1) \times 10^3$
	+H5	$(1.0 \pm 0.1) \times 10^4$	1.8 ± 0.1	$(1.8 \pm 0.4) \times 10^5$

4. Discussion

N-glycosylation is a key post-translational process that ensures a proper folding and intracellular traffic, increases stability, reduces the sensitivity to proteases immunogenicity, and finally may increase half life of proteins (Brooks *et al.*, 2013; Helenius *et al.*, 2004; Sinclair *et al.*, 2005). Therefore, this modification is crucial for most membrane and secreted proteins. Recent studies have confirmed the importance of this modification for a correct and/or specific function of multiple haemostatic proteins, including platelet and coagulation cascade proteins (Gils *et al.*, 2003; Ni Ainle *et al.*, 2011; Preston *et al.*, 2013). In this report we fulfill previous studies demonstrating that mutations in *SERPINC1* generating new N-glycosylation sites in antithrombin might cause all possible types of antithrombin deficiencies. Thus, N-glycans in key structural regions, like S82N on the shutter (Fitches *et al.*, 2001) affects the correct folding of antithrombin leading to intracellular polymerization or degradation. The final result is a reduced secretion rate that explains the type I deficiency observed in carriers. However, other mutations may introduce new N-glycans in antithrombin that do not significantly perturb the folding and/or secretion of the variant protein, but impair the function of this key anticoagulant. Accordingly, the presence of an N-glycan in the HBS does not hinder the interaction of the serpin with the target protease, but significantly impairs the

interaction of antithrombin with its cofactor heparin and the subsequent activation of the serpin. Mutations, as the I7N, explain the type IIb deficiency observed in carriers (Brennan *et al.*, 1988). The relevance that N-glycosylation has on heparin affinity is also supported by the role of the N-glycan at N135, which defines the two main glycoforms of antithrombin observed in plasma (McCoy *et al.*, 2003; Turk *et al.*, 1997).

Our study is the first one that has evaluated the role of an N-glycan at the RCL of antithrombin, reaching interesting results that improves the knowledge of this anticoagulant serpin and generates interesting conclusions that might be extrapolated to other inhibitory serpins. We have demonstrated that mutations creating a consensus sequence for N-glycosylation at different positions of the RCL of antithrombin (P2 and P10) are efficiently glycosylated. Furthermore, these new N-glycans do neither significantly affect the correct folding of the protein nor the secretion of the antithrombin variants. So as it could be expected, N-glycans at the RCL affect the reactivity of antithrombin, causing a type IIa deficiency.

This impairment in the reactivity of mutants is driven by two different mechanisms depending on the position of the N-glycan. The presence of the N-glycan at P10, far from R393 (P1), does not significantly impair the first step, which is the recognition of the proteases. Therefore, the protease may execute its catalytic activity on the variant of antithrombin, cleaving the P1-P1' bond. However, the new N-glycan slows the insertion of the cleaved RCL into the central A sheet, as covalent complexes are not formed and antithrombin behaves as a substrate. Natural mutations affecting the same or close residues that do not generate N-glycosylation sequences also behave as substrate, but a proportion of these variants is still able to inhibit the target proteases (Mushunje *et al.*, 2003).

In contrast, the presence of an N-glycan close to the residue attacked by the target proteases (P1) generates a steric hindrance that does not allow initial encounter. Actually, our results show that the N-glycan hampered the interaction with thrombin and covalent complexes were not generated, in absence of heparin. But, this defect is partially reverted by the heparin activation; despite the rates of inhibition is severely reduced (10,000-fold). Therefore, we think that the conformational changes trigger by heparin might condition the disposition of the N-glycan at N392 (Huntington, 2003; Schedin-Weiss *et al.*, 2010). This result also demonstrates that the presence of an N-glycan at P2 is not a significant obstacle for a correct insertion of the cleaved RCL, as

the P2 residue does not participate of the new s4A, unlike the P10 residue (A384) (Figure 3).

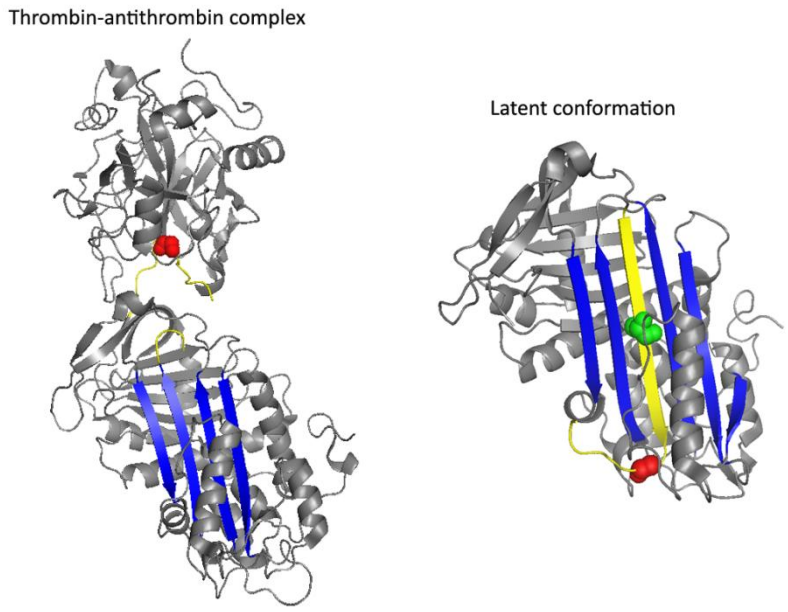


Figure 3. Ribbon diagram of thrombin-antithrombin complex (PDB code 1TB6) and latent antithrombin (2BEH) showing the localization of the P2 (red spheres) and P10 (green spheres) residues. The RCL is shown in yellow and central A strands are shown in blue. All structures were rendered in Pymol (www.pymol.org).

Surprisingly, we found that the N392 variant was able to efficiently inhibit FXa even in the absence of heparin; although presenting stoichiometry values greater than control antithrombin (4.9 vs 1.3, respectively). Thus, this variant showed rates of FXa inhibition similar to control antithrombin. These data support that an N-glycan in P2 may regulate the specificity of antithrombin for proteases. Actually, the role of P2 residue in the interaction or specificity of antithrombin with its target proteases has been already described (Chuang *et al.*, 2001b; Yang *et al.*, 2009). Our data could also help to define protease specificity in other serpins. In the case of antithrombin, many efforts have been driven to generate molecules with specific inhibitory activity for only one protease. We point out the excellent studies of Olson and coworkers, who identified exosites that promote a rapid inhibition of factors Xa and IXa in antithrombin activated by heparin (Dementiev *et al.*, 2013; Izaguirre *et al.*, 2006; Izaguirre *et al.*, 2003). The use of such exosites by serpins in the recognition of their target proteases has been studied in other serpins, as kallistatin and heparin cofactor II (Chen *et al.*, 2000; Van Deerlin *et al.*, 1991). Additionally, the generation of an antithrombin able to inhibit FXa, but with

restricted reactivity against thrombin, using a diferent strategy (Izaguirre et al 2009) may have useful therapeutic applications that must be explored in future studies. Due to this variant could have reduced bleeding side effects in comparison with wild-type antithrombin. Moreover, the presence of an additional glycan could also increase the half-life of this protein (Elliott *et al.*, 2003; Sinclair *et al.*, 2005), which may improve the pharmacokinetic effectiveness of antithrombin supplementation in patients.

In summary, the generation of new N-glycosylation sites might be used as a new tool for the study of antithrombin and the whole serpin superfamily. N-glycans in RCL affect the reactivity of antithrombin and may determine specificity of protease. Analysis of N-glycosylation on the serpin superfamily revealed a high concentration of N-glycans close to the RCL (Figure 4), which might contribute to protease specificity. Finally, these recombinant molecules could have interesting therapeutic uses.

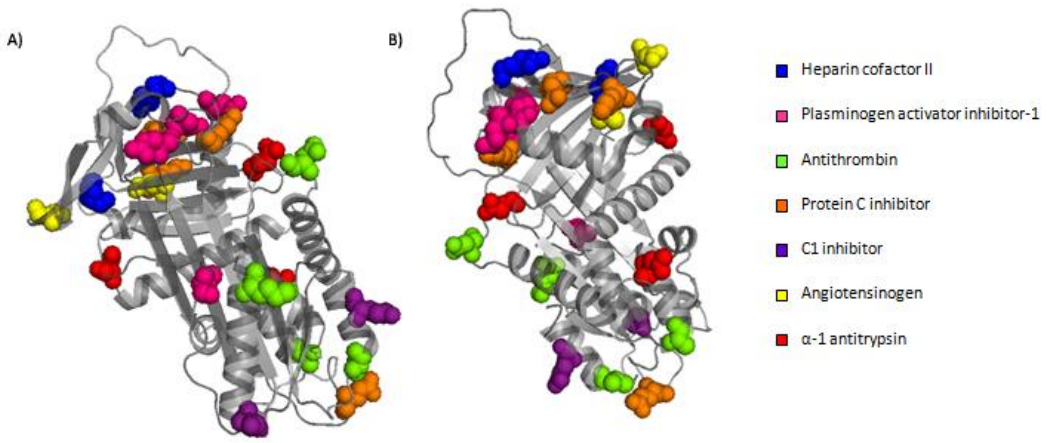


Figure 4. Ribbon diagram of α-1 antitrypsin, the archetypal serpin (A) and view of the 180º turned image (B) (PDB code: 3NE4). The localization of N-glycosylation sites in different serpins is displayed with spheres. All structures were rendered in Pymol (www.pymol.org).

GLOBAL DISCUSSION

The first reference to antithrombin was noticed at the end of the 19th century when A. Schmidt described that thrombin is gradually inhibited when added to defibrinated plasma by the presence of a specific inhibitor of the enzyme, that P. Morawitz named antithrombin. Since then, multiple evidences support the key role of antithrombin in hemostasis and thrombosis, and hundreds of studies have investigated this crucial anticoagulant. Despite such an intense research, the biological and clinical relevance of this fascinating molecule still encourage an intense research on this old but crucial hemostatic molecule. Actually, antithrombin is the most important physiological inhibitor of blood coagulation proteases. Hence, the complete deficiency of antithrombin cause embryonic lethality and plays an important role in regulation of blood coagulation in the myocardium and liver (Ishiguro *et al.*, 2000). Moreover, inherited antithrombin deficiency is a major genetic factor for thrombosis that causes a 10-20-fold increase in the risk of venous thromboembolism. (van Boven *et al.*, 1997; Yang *et al.*, 2010) Actually, the prevalence of antithrombin deficiency in the general population is 1:500 to 1:3,000 and it is found in 2–5% of patients with venous thrombosis (Luxembourg *et al.*, 2011; Perry *et al.*, 1996; Rosendaal, 1999; Rossi *et al.*, 2008)

Aiming to find new information on antithrombin, our study was originally based on the analysis of cases with antithrombin deficiency. Actually, the study of natural mutants has helped to identify functional domains of antithrombin, such as the reactive center loop and heparin binding site and has helped to identify mechanisms associated with the associated clinical phenotype (Blajchman *et al.*, 1992; Corral *et al.*, 2007; Chang *et al.*, 1986; Koide *et al.*, 1984; Raja *et al.*, 2003). During the last 10 years our group has collected samples from 142 cases with antithrombin deficiency, mainly from Spain. One hundred and sixteen out of 142 patients with thrombosis and antithrombin deficiency had mutation or gross deletions. The analysis of these natural mutations has revealed new information about this key anticoagulant and has opened new field of research.

We have also used mutations generated in the laboratory to evaluate the role of certain mutations or regions on antithrombin function. Figure 12 sumarizes the mutations we have evaluated in this study.

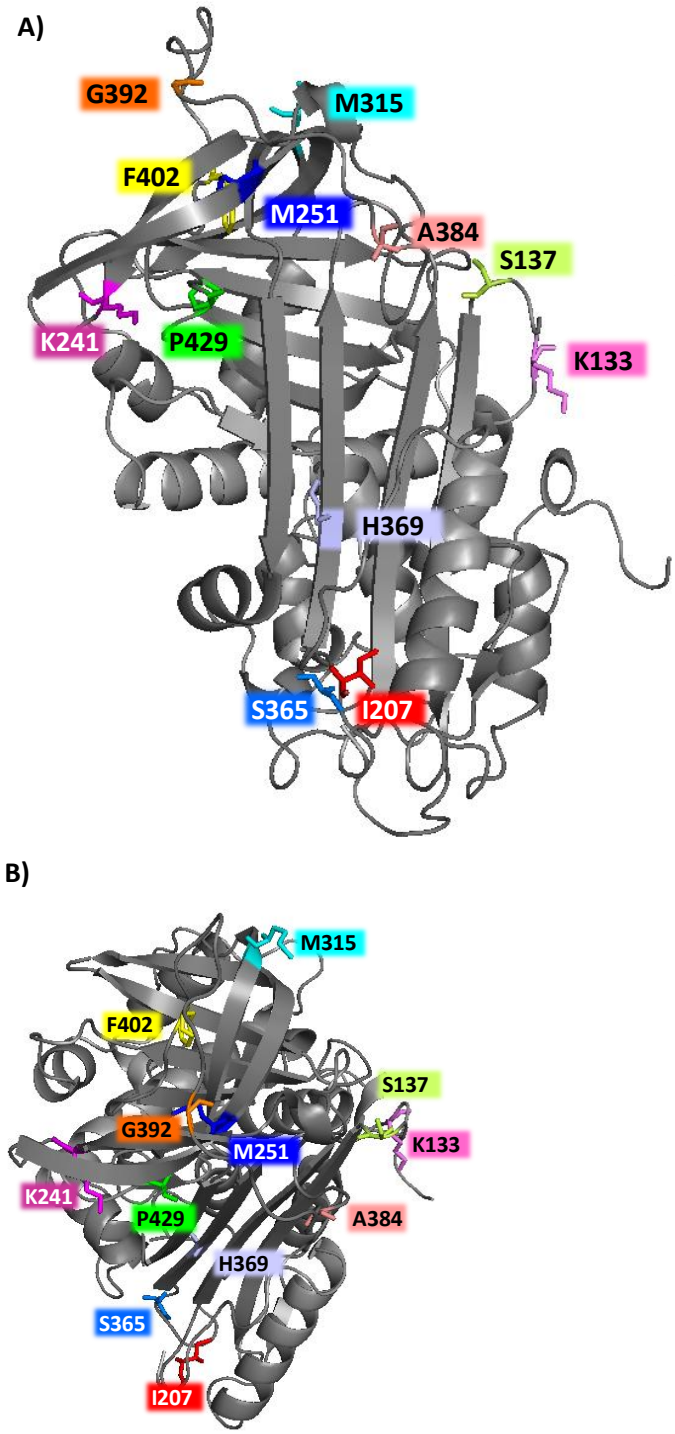


Figure 12. Ribbon diagram of native antithrombin (PDB code 1T1F). A) Stick representation of the localization of residues that have been mutated and evaluated in this study. A384 is displayed in salmon, G392 in orange, M315K in cyan, F402 in yellow, M251 in blue, K241 in magenta, P429 in green, S137 in lemon, K133 in pink, H369 in lightpurple, S365 in marine and I207T in red . B) The ribbon diagram provides a close-up of the C-sheet viewed from the top of the reactive loop. All structures were rendered in Pymol (www.pymol.org).

Compound heterozygosis: frequency and prognostic importance.

The risk of thrombosis of subjects with antithrombin deficiency is high, but a significant clinical heterogeneity has been observed, even between carriers of the same family. Our group has investigated mechanisms involved in such variability finding mechanisms associated with mild thrombotic phenotype (Martinez-Martinez *et al.*, 2012c) but also with severe clinical manifestations (Martinez-Martinez *et al.*, 2012b). In this study, we

have identified a new mechanism associated with a worse prognosis. Two out of 96 cases with antithrombin deficiency had compound heterozygosity in the *SERPINC1* gene. In both cases, the A384N mutation, a relatively frequent mutation in the Spanish population that mildly increases the risk of venous thrombosis by an inefficient inhibition of thrombin, coexisted with a point mutation, which affected severely to the function of the protein (Corral *et al.*, 2004; Mushunje *et al.*, 2003). The presence of functional mutations in both alleles of the *SERPINC1* gene reduced the anticoagulant activity, particularly the anti-IIa activity, thus increasing the risk or severity of thrombosis. Thus subjects with compound heterozygosis have early or recurrent thrombosis. This result has prognostic relevance, which has to be taken into account due to the relatively high incidence of compound heterozygosis identified in our serie (2% of cases with antithrombin deficiency). But our study could also have a significant impact on the diagnostic, particularly in familial studies. Since the A384S mutation is not detected by anti-Xa tests or antigen determination (Corral *et al.*, 2004), and familial studies currently focused on detecting antithrombin deficiency by means of functional tests or the identification of the index mutation by molecular methods, compound heterozygosity involving A384S mutation might be underestimated. Our results encourage evaluating the Cambridge II mutation in familial studies, even if the proband does not carry this mutation.

This result opens new perspectives to the analysis of other mild mutations affecting *SERPINC1*, and the possibility of being in compound heterozygosis with strong mutations. We have just identified a similar situation described here for the Cambridge II mutation for other variant; Antithrombin Dublin: a high incidence of compound heterozygosis in patients with severe clinical phenotype and antithrombin deficiency (Navarro et al, submitted).

Identification of new functional regions of antithrombin

Two main functional domains are classically described in antithrombin: the RCL, involved in the recognition of the target protease and the mechanism of its inhibition, and the HBS, involved in the activation of the anticoagulant capacity of antithrombin. The aim of this study was to identify new regions and crucial residues for an efficient mechanism of inhibition out of these classical functional domains of antithrombin. Our study was initiated by selecting patients with venous thrombosis and antithrombin

deficiency that carried mutations causing a type II deficiency that located out of these functional domains.

In our serie of patients with antithrombin deficiency, 51 cases had type II deficiency, 45 caused by missense mutations. Forty cases carried missense mutations affecting established functional domains (23 at the HBS, 9 at the RCL, 4 at the C-sheet, 4 at the signal peptide). Five cases have missense mutations not clearly located in functional domains: I207T; L341F; S349P; S365L and H369Y.

Identification of a new region involved in the insertion of the cleaved RCL

We considered that mutations of residues potentially interacting with the RCL during its insertion might be good candidates to explain inactive variants. To validate this hypothesis, we studied two cases carrying I207T and S365L, because these residues, despite of being distant in the primary sequence, they were structurally close and formed a region where the internalized RCL exit from the central A sheet in the latent and cleaved conformations. Moreover, H369Y was also studied since it might interact with the new s4A sheet formed by the inserted RCL.

The analysis of plasma antithrombin in carriers of these mutations revealed increased levels of hyperstable and inactive conformations of this serpin: disulphide dimers (S365L), lower denaturation temperature of native antithrombin (I207T) or latent antithrombin (H369Y) supporting that these mutations may have conformational consequences, which certainly contribute to the deficiency observed in carriers. But these results did not explain the amount of inactive variant found in plasma. We investigated the mechanism making these variants inactive, by using recombinant proteins. Our studies demonstrated that a proportion of these three variants behaved as a substrate for target proteases, in the cases of S365L mutant, did not form complexes, since all protein behaved as substrate. This result and the localization of these residues in crucial check-points of the RCL internalization process, suggest a new functional region in antithrombin.

Serpins share a common mechanism of protease inhibition and a molecular architecture based on a more stable five-stranded A β-sheet. The first step of this mechanism, the formation of an acyl-intermediate complex, has a critical importance because of its irreversible nature. Specific mutations may decrease the rate of loop insertion relative to deacylation to lead free protease and cleaved serpin. Nowadays, it has been described that certain mutations on the RCL and the shutter region of serpins might have this

effect, behaving serpins as substrates (Blajchman *et al.*, 1992; Corral *et al.*, 2007; Hansen *et al.*, 2001; Lane *et al.*, 1997; Raja *et al.*, 2003). Our study reveals that residues that interact with the cleaved RCL during its insertion into the A-sheet seem to have the same relevance than residues affecting the hinge region of the RCL for an efficient inhibition of proteases. Thus, mutations affecting these residues may delay the insertion of the RCL, which may facilitate the release of the target protease.

It is possible that additional missense mutations potentially affecting residues interacting with the cleaved RCL and/or the protease during its insertion and translocation may have similar consequences.

C-sheet of antithrombin: a new region relevant for a correct maturation of the N-glycans that is involved in the heparin affinity

The identification of an abnormal glycosylation in a pleiotropic mutant (K241E) that explained the impaired heparin affinity and the mild risk of thrombosis in carriers encouraged to evaluate the effects of different natural pleiotropic mutations on the glycosylation of antithrombin and their functional effects. We selected 5 pleiotropic mutations identified in patients with antithrombin deficiency and located at each one of the strands of the C-sheet (K241E, M251I, M315K, F402L, and P429L). Analysis of glycoforms of recombinant mutants revealed that all pleiotropic mutants, except for M315K that affects a non-exposed residue, presented two glycoforms (variant 1 and variant 2). Variant 1, with abnormal glycosylation, had reduced heparin affinity and severely affected the reactivity with the target proteases. Our data support that not only fucosylation, which has been demonstrated that impairs the heparin affinity (Martinez-Martinez *et al.*, 2010), but also additional N-glycosylation modifications influence the heparin affinity of this anticoagulant. In contrast, variant 2, with similar electrophoretic mobility and heparin affinity to wild-type antithrombin, had an impaired inhibitory activity that was partially compensated by the activation with heparin. Our results suggest the C-sheet of antithrombin as a new region relevant for a proper maturation of the *N*-glycans. Therefore, pleiotropic mutations lead to defects on the maturation of the N-glycans that is responsible for the reduced heparin affinity. Moreover, it may also help to explain the different clinical phenotype in patients carrying any of these pleiotropic mutations. Thus, mutants with glycoforms able to be activated by heparin are expected to have milder clinical phenotype. In contrast, F402L variant did not recover the anticoagulant activity after activation with heparin, which might also

explain the severe clinical phenotype reported for patients carrying this mutation (Emmerich *et al.*, 1994a; Lane *et al.*, 1992). As it has been demonstrated that T401 directly interacts with E37 of FXa (Johnson *et al.*, 2006b), we consider that F402 might be relevant for the interaction with the target proteases and the mutation F402L might impair the interaction between these residues even in the activated conformation.

The analysis of type II deficiencies has allowed us to identify two new functional regions in antithrombin. Mutations affecting residues of these new regions have pathological consequences, as they reduce the anticoagulant capacity by different mechanisms. The analysis of these cases may also assist to identify new functional regions of this key anticoagulant.

Glycosylation of antithrombin: new insights for antithrombin deficiency and a model for studying this key post-translational process

Antithrombin is an N-glycoprotein with 4 potential N-glycans that play a role not only in the half-life of the molecule, but also in the heparin affinity of this protein (McCoy *et al.*, 2003; Ni *et al.*, 2000; Picard *et al.*, 1995). Moreover, aberrant glycosylation may also have pathological relevance, as we have demonstrated for different pleiotropic mutations. Therefore, the analysis of this post-translational modification in antithrombin is an emerging field of interest.

Mutations creating new glycosylation sites and antithrombin deficiency

We demonstrated that an abnormal maturation of the N-glycan, induced by mutations in the C-sheet, might influence the heparin affinity and contribute to explain certain cases with antithrombin deficiency (type IIc or pleiotropic). However, incomplete glycosylation of this anticoagulant might also have pathological consequences. Mutations inducing increased and reduced number of N-glycans in antithrombin have also been associated with antithrombin deficiency. Thus, the N135T mutation, identified in a family presenting borderline deficiency and unclear thrombotic consequences, removes this glycosylation site generating a variant protein similar to β-antithrombin (Bayston *et al.*, 1999). On the other hand, three reports have shown different mutations in *SERPINC1* that, generating new N-glycans, also cause type I, IIb and IIc antithrombin deficiency (Brennan *et al.*, 1988; Fitches *et al.*, 2001).

We aimed to investigate whether type IIa deficiency might also be caused by mutations introducing an N-glycan in the reactive centre loop (RCL) of antithrombin. We

designed two mutations affecting residues close to or far from the reactive P1 (Arg393) residue (G392N and A384N, respectively). Moreover, this study might also show the effect of an N-glycan at the RCL on the secretion and function of antithrombin. We used recombinant variants produced in HEK-EBNA cells. Our results suggest that these RCL mutations are efficiently glycosylated. The presence of a new N-glycan in the RCL of antithrombin did not affect the folding and secretion of antithrombin but modified its anticoagulant activity through two different mechanisms depending on the distance of the N-glycan to the reactive R393 (P1). Thus, A384N (P10) did not influence the interaction with proteases, but affected the insertion of the cleaved RCL into the A-sheet, and the variant behaved as a substrate.

In contrast, the glycan on N392 (P2) blocked the interaction with thrombin, in native state, as neither thrombin-antithrombin complex nor cleaved antithrombin was observed. However, the steric hindrance of an N-glycan at P2 was eliminated by activation with heparin, and the cleaved RCL was correctly inserted into the A-sheet, forming covalent complexes. Interestingly, the N-glycan at P2 did not significantly impair the inhibition of FXa, even in absence of heparin. Therefore, N-glycans on P2 of the RCL defines certain specificity to proteases.

Recent studies show the significance of glycosylation for a correct and/or specific function of multiple haemostatic proteins, including platelet proteins and clotting factors (Gils *et al.*, 2003; Ni Ainle *et al.*, 2011; Preston *et al.*, 2013). Our group has contributed to demonstrate that abnormal N-glycosylation of antithrombin may also have pathological consequences through a myriad of different mechanisms, from aberrant glycosylation (Martinez-Martinez *et al.*, 2010) to defective glycosylation (de la Morena-Barrio *et al.*, 2012b). Moreover, *SERPINC1* mutations creating new *N*-glycosylation sites may have pathological consequences. In this report we fulfill previous studies demonstrating that mutations in *SERPINC1* generating new N-glycosylation sites in antithrombin might cause all possible types of antithrombin deficiencies. This study demonstrates that mutations generating new N-glycans at the RCL of antithrombin will probably cause a type IIa deficiency.

Moreover, our report shows that the generation of new N-glycosylation sites may be a new tool for the study of antithrombin and other serpins and opens new possibilities for anticoagulant therapy.

Antithrombin as a model to study N-glycosylation

Two glycoforms of antithrombin, a key anticoagulant serpin, are identified in plasma: α and β, with four and three N-glycans, respectively, due to an inefficient glycosylation at N135 by still unknown mechanisms (McCoy *et al.*, 2003; Picard *et al.*, 1995). The lack of this N-glycan significantly increases the heparin affinity of the β-glycoform (McCoy *et al.*, 2003; Picard *et al.*, 1995). Recent and very interesting studies from the same group have demonstrated that an aromatic sequon (Phe-Y-Asn-X-Thr) in reverse β-turns increases the N-glycosylation efficiency and global stability of proteins (Culyba *et al.*, 2011; Price *et al.*, 2012; Price *et al.*, 2011). We evaluated the effect of the aromatic sequon in this inefficient glycosylation site of antithrombin, despite of being located in a loop. We analyzed the biochemical and functional consequences of different mutations affecting the glycosylation of the N135 residue, including the generation of an aromatic sequon: K133F, S137T and K133F/S137T. HEK-EBNA cells transfected with wild-type plasmid (K133-Y-N135-X-S137) generated 50% of α and β-antithrombin, while K133F, S137T, and the double mutant (K133F/S137T) had improved glycosylation efficiency, leading to the secretion of only α-antithrombin, according to electrophoretic and mass analysis. The presence of the aromatic sequon did not affect the global stability of this conformational sensitive serpin as revealed by calorimetry. However, it stabilized the native conformation, since it impaired the activation induced by heparin as shown by rapid kinetics of heparin binding and equilibrium dissociation constant studies. Accordingly, K133F and K133F/S137T mutants have a reduced anticoagulant activity, being more severe in the double mutant. Our data support that the aromatic sequon improves completely the efficiency of N-glycosylation and partially the stability of proteins in a different structural context from reverse turns.

Antithrombin specificity to proteases

Our data could also help to reach an old challenge in serpins: to define protease specificity. In the case of antithrombin, many efforts have been driven to generate molecules with specific inhibitory activity for only one of its target proteases, particularly FXa and thrombin. This work has identified two mechanisms potentially involved in such specificity.

Mutations in residues differentially interacting with FXa and thrombin may impair the inhibitory activity over one of these proteases. These residues have been searched at the RCL and C-sheet, based on the initial protease-serpin interactions. Additionally, Cambridge II variant has impaired anti-thrombin activity in presence of unfractionated heparin, although the anti-FXa is not affected (Mushunje *et al.*, 2003). Nevertheless, our results extend the search to new regions, and suggest that interactions in the last steps of the inhibitory mechanism, when the protease is being dragged to the opposite pole of the serpin are also candidates for getting this specificity. Our data suggest that I207 may be one of these residues. Mutation of this residue to threonine affects more severely the reactivity with FXa than with thrombin.

Moreover, N-glycans at the RCL, like those generated at P2, might efficiently inhibits FXa but not thrombin in native state.

Using these strategies, we might design antithrombin molecules able to inhibit FXa, but with restricted activity against thrombin. These molecules may have the same useful therapeutic applications than low molecular weight heparins (Hull *et al.*, 1992), with reduced bleeding side effects in comparison with wild-type antithrombin and avoiding the side effects caused by low molecular weight heparins. Moreover, we might combine these designs with the incorporation of additional N-glycans increasing the half-life of the molecule.

CONCLUSIONS

1. **Compound heterozygosity involving the A384S mutation is relatively frequent among patients with antithrombin deficiency. This state significantly impairs the anticoagulant capacity and increases the risk of venous thrombosis and the severity of clinical manifestations. Moreover, the failure of usual thrombophilic tests to detect A384S mutation, encourage evaluating potential compound heterozygosis involving A384S in familial studies, even if the proband does not carry this mutation.**

2. **The opening for the internalized RCL to exit from the central A-sheet is a key new region for the reactive center loop insertion. Mutations in this region affect to the dragged of the covalently bound protease to the opposite end of antithrombin for the complete inhibition.**

3. **The C-sheet is a relevant region for the correct maturation of N-glycans on antithrombin. The aberrant glycosylation induced by mutations on residues of the C-sheet reduces the heparin affinity.**

4. **The introduction of the aromatic sequon (Phe-X-Asn-Y-Thr) on N135 of antithrombin improves the efficiency of N-glycosylation hindering the changes induced by heparin. These results support that this aromatic sequon may be used for an efficient glycosylation in structural contexts other than reverse turns.**

5. **Mutations introducing new glycans on the reactive center loop of antithrombin are correctly glycosylated and the variants are secreted, but interfere in the reactivity. The N392 variant determines specificity of protease, since this variant properly inhibits FXa but not FIIa.**

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SUMMARY/RESUMEN

Introducción

El sistema hemostático tiene la función de evitar las consecuencias letales que tendría la hemorragia cuando se produce un daño endotelial. Esta función la realiza a través de una serie de proteínas plasmáticas que forman la cascada de la coagulación y las plaquetas. La exposición de factor tisular desencadena una activación seriada de diversas serin proteasas, lo que culmina con la generación de trombina. La trombina, es una serin proteasa procoagulante crucial puesto que es responsable de la formación de la malla de fibrina, pero también es el principal agonista plaquetario. Muchas de estas enzimas procoagulantes están reguladas por serpinas (inhibidores de serin proteasas). Las serpinas comparten una estructura conservada que consta de tres láminas β (A–C) y nueve hélices α (A–I), que se organizan en una conformación metaestable (que no es la conformación termodinámicamente más favorable), proveyéndolas de una flexibilidad estructural. Esta flexibilidad afecta particularmente al centro reactivo (RCL), implicado en la interacción específica con las proteasas diana; y la lámina A central. Esta configuración estructural es necesaria para la formación del complejo con la proteasa diana y eficiente mecanismo inhibitorio. La acción proteolítica de la proteasa sobre el RCL de la serpina libera energía que permite la transición de la serpina a una conformación hiperestable, con la inserción del RCL roto dentro de la lámina A, en un proceso en el cual arrastra a la proteasa y la inhibe. Es por tanto, un mecanismo inhibitorio por suicidio, ya que este proceso es irreversible.

La antitrombina es el principal anticoagulante endógeno. Sus principales dianas son el factor X activo (FXa) y la trombina o factor II activo (FIIa). Dicha serpina presenta, como particularidad respecto a otras serpinas, un RCL parcialmente insertado en la lámina A. La antitrombina se sintetiza en el hígado y alcanza en plasma niveles en torno a 150 µg/ml. Tiene una vida media aproximada de 3 días. Se trata de una molécula de 58-56 KDa. Está compuesta por 432 aminoácidos y presenta cuatro sitios de N-glicosilación (N96, N135, N155, N192) y tres potenciales puentes disulfuro. La función anticoagulante de la antitrombina está potenciada por glicosaminoglicanos (GAGs) que se localizan en el endotelio de los vasos, que actúan como cofactores. Los GAG y se unen al sitio de unión a heparina (HBS). El HBS está formado por la hélice D, la hélice A y el extremo N-terminal de la antitrombina. Dicha unión provoca cambios conformacionales que llevan a la expulsión completa del RCL. Este proceso condiciona un espectacular incremento de la actividad inhibitoria. Por lo tanto, gracias a este

mecanismo de activación se restringe la actividad anticoagulante de la antitrombina a la zona vascular dañada y es la base de la acción anticoagulante de las heparinas terpeúticas. En el caso concreto de la inhibición de trombina, para alcanzar el grado máximo de activación se requiere la formación de un complejo ternario antitrombina-trombina mediante heparina no fraccionada (complejo de Michaelis). Mientras que con el FXa, la unión de pentasacárido (cinco unidades de monosacárido necesarias para la activación) es suficiente para incrementar significativamente la actividad inhibitoria de la antitrombina.

Por otro lado, debido a su complejo y preciso mecanismo inhibitorio, las sepinas son muy sensibles, incluso pequeñas modificaciones, como mutaciones puntales. Por ello, el cambio de un solo aminoácido puede provocar o facilitar el plegamiento de la serpina a una configuración hiperestable no inhibitoria: la forma latente, con el RCL insertado en la lámina A de la propia molécula, o los polímeros, con el RCL insertado en otra molécula. La formación de polímeros tiene consecuencias patológicas, las denominadas serpinopatías, que incluyen ciertas neuropatías, enfisemas, cirrosis y trombosis. El mecanismo de polimerización de serpinas *in vivo* no está completamente elucidado y no existen fármacos eficientes capaces de inhibir este proceso. En este sentido, la antitrombina presenta una particularidad con respecto al resto de serpinas, es que está molécula puede formar dímeros cerrados unidos por puentes disulfuro. Por otro lado, su configuración nativa con el RCL parcialmente insertado hace que la antitrombina sea más sensible a la transformación a latente. De hecho, incluso de forma fisiológica, la antitrombina se transforma espontánea y lentamente a forma latente. Constituyendo el 5% de la antitrombina plasmática.

El papel clave de la antitrombina en la cascada de la coagulación queda reflejado en el alto riesgo de trombosis asociado a la deficiencia en heterozigosis de este anticoagulante (OR: 10-20). Además, la deficiencia completa de antitrombina provoca letalidad embrionaria. Sólo se han descrito algunos casos en homozigosis o heterozigosis compuesta, los cuales están asociados con un alto riesgo de trombosis y de recurrencia. La mayoría de los casos con deficiencia congénita de antitrombina se deben a alteraciones s en el gen que codifica la antitrombina, *SERPINC1*. Existen más de 250 mutaciones descritas en este gen. Las consecuencias de estas mutaciones en los niveles antigénicos y la función de la antitrombina definen los tipos de deficiencia de antitrombina: tipo I, si no se detecta forma mutada en el plasma, o tipo II, cuando la forma mutada, con menor o nula actividad inhibitoria es secretada y localizada en

circulación. Dentro de las deficiencias tipo II hay tres subgrupos: i) tipo IIa o del RCL, que afectan a la reactividad de la proteína, ii) tipo IIb o del HBS, que disminuyen la afinidad por heparina, iii) tipo IIc o pleiotrópicas, localizadas en la lámina C y con múltiples efectos.

La antitrombina presenta una única modificación post-traduccional, la N-glicosilación. Dicha modificación se produce durante el plegamiento de la proteína en el retículo endoplasmático sobre las asparraginas que se encuentran en contexto de una secuencia consenso concreta (Asn-X-Ser/Thr/Cys) y en la conformación adecuada para que actúe la oligosacariltransferasa (OST). La N-glicosilación es importante en esta proteína porque afecta a su funcionalidad. Como hemos indicado anteriormente, la antitrombina contiene cuatro sitios de N-glicosilación, uno de los cuales (N135) no es eficientemente glicosilado, sólo se glicosila en el 50% de las ocasiones. Esta ineficiente glicosilación es la responsable de la existencia en plasma de dos glicoformas: α-antitrombina, con cuatro N-glicanos y que es la forma mayoritaria (90%), y β-antitrombina, con tres glicanos que es la forma minoritaria en plasma (10%). Los diferentes niveles de α- y β-antitrombina se deben a la diferente tasa de aclaramiento. Debido a que la ausencia de glicosilación en N135 por su cercanía al HBS, provoca el aumento la afinidad por heparina de la glicoforma β. Finalmente, la alteración en la glicosilación de antitrombina, como la adición de fucosa en los glicanos de antitrombina, reducen la afinidad por heparina. La fucosilación de esta serpina se ha detectado en la expresión recombinante en células BHK y CHO.

Objetivos

1) Caracterización de nuevos factores de riesgo trombótico asociados con la deficiencia de antitrombina.
2) Identificación de nuevas regiones implicadas en la funcionalidad de la antitrombina.
3) Evaluación del proceso y papel de la N-glicosilación de antitrombina

Resultados

Objetivo 1.

Heterozigosis compuesta en la deficiencia de antitrombina implicando la mutación Cambridge II (A384S)

El riesgo de trombosis en los portadores de deficiencia de antitrombina es elevado, pero se ha observado una gran heterogeneidad clínica, incluso en los portadores de la misma familia. En este estudio hemos identificado heterozigosis compuesta en el gen *SERPINC1*, en 2 de 96 casos con deficiencia de antitrombina. Ambos casos, comparten la mutación A384S, responsable de la variante Cambridge II, una mutación relativamente frecuente en población española que incrementa de forma moderada el riesgo de trombosis venosa debido a una ineficiente inhibición de trombina. Esta mutación coexiste con una mutación puntual, no descrita previamente y distinta en los dos casos identificados. El primer paciente (P1) además presentaba la mutación c.1190C>T, que causa el cambio de una serina por treonina en posición 365, un residuo conservado localizado en la hebra 5A. P1 es un hombre de 41 años que desarrollo tromboembolismo pulmonar y trombosis venosa profunda a los 38 años. Cinco de los 7 miembros de la familia materna presentaron también esta mutación, pero no la mutación Cambridge II, y trombosis venosa. Además, el sujeto presentó dímeros unidos por puente disulfuro y un 76% de antígeno. Por lo tanto, esta mutación tiene efectos conformacionales, lo cual ayuda a explicar la severidad trombótica observada en los portadores.

El segundo paciente (P2) es una mujer de 33 años que desarrolló trombosis venosa profunda a los 31 años. Además de la mutación Cambridge II, es portadora de la mutación c.409 -2A>T, afectando la señal donadora del procesamiento del intrón 2, lo cual se traduce en una deficiencia de antitrombina tipo I, ya que sus niveles antigénicos fueron del 52%. En ninguno de los probandos, ni en sus familias hubo ningún otro factor de riesgo trombofílico. En los dos casos de heterozigosis compuesta la actividad anti-IIa se encontraba significativamente más reducida que en los portadores de la mutación severa, lo que justifica la mayor severidad clínica de estos casos.

Estos resultados no solo tienen una relevancia pronóstica, sino que también tienen un impacto diagnóstico, particularmente en estudios familiares, ya que la mutación A384S no se detecta por las pruebas funcionales o antigénicas que actualmente se emplean rutinariamente para diagnosticar la deficiencia de antitrombina. Además, los estudios

moleculares familiares, normalmente se restringen a la mutación identificada en el probando. Por todo ello, y por la elevada frecuencia identificada en nuestra serie, es posible que la mutación Cambridge II, especialmente en heterozigosis compuesta, esté infraestimada.

Objetivo 2.

Identificación de nuevos regiones implicadas en la funcionalidad de antitrombina

La identificación de dominios funcionales relevantes para la antitrombina se ha realizado, en parte gracias a la identificación de mutantes naturales en pacientes con deficiencia de antitrombina. Con el objetivo de identificar nuevos residuos o regiones funcionales relevantes en el desarrollo de deficiencias de este potente anticoagulante, nuestro grupo caracterizó casos con deficiencia tipo II asociada a mutaciones localizadas en regiones no incluidas en dominios ya establecidos: RCL y HBS.

a) Identificación de residuos implicados en la inserción del RCL

En este caso, nos centramos en la zona opuesta al RCL, debido a que identificamos dos mutaciones en pacientes con deficiencia de antitrombina, cercanas estructuralmente (S365L & I207T). Curiosamente estas dos mutaciones forman la puerta de salida del RCL en la lámina A. Debido a la limitación del estudio de muestras de portadores, tanto por la escasa cantidad de plasma disponible, como por la presencia de formas silvestres de antitrombina en el plasma al estar en heterocigosis, decimos generar las proteínas recombinantes. Así, el mutante S365L formaba mayores cantidades de dímeros unidos por puente disulfuro que forma la silvestre de antitrombina y las formas monoméricas no eran capaces de formar complejos con trombina, ni FXa, comportándose como sustrato.

En cambio, la variante T207 mostró una afectación de la velocidad de inhibición de FXa en presencia de pentasacárido, mientras que la inhibición de FIIa prácticamente no se vio alterada. Por lo tanto, dicha mutación afecta diferencialmente la inhibición de ambas proteasas diana de antitrombina. Este resultado es similar, aunque opuesto al que se observa con la variante Cambridge II (A384S), ya que esta mutación afecta a la inhibición del FIIa en presencia de heparina y no a la inhibición del FXa. Además, el mutante I207T presentaba cierta inestabilidad conformacional, puesto que su temperatura de desnaturalización era menor que la silvestre.

Estos resultados sugieren que el cambio S365L como el I207T dificultan la internalización del RCL cuando la proteasa está covalentemente unida y es translocada

al polo opuesto de la serpina. Aunque con la particularidad, de que en el caso del mutante I207T, solamente la inhibición del FXa está afectada, y no la del FIIa, al igual que lo observado en la pruebas funcionales con el plasma del probando. Por lo tanto, estos resultados animan al desarrollo de nuevos estudios para identificar otros residuos en la misma zona u otras regiones de la antitrombina que interfieran en el mecanismo de internalización del RCL.

b) Papel de la lámina C en la maduración de los glicanos de antitrombina: relevancia funcional de las mutaciones pleiotrópicas.

Un trabajo previo de nuestro grupo demostró que una mutación pleiotrópica (K241E) en un paciente con trombosis, alteraba el proceso de glicosilación de la antitrombina, provocando una glicosilación aberrante (fucosilación) que provocaba la reducción de la afinidad por heparina. Debido a este estudio previo, a la escasa información sobre el mecanismo de este tipo de deficiencias y a la enorme heterogeneidad clínica de portadores de mutaciones pleiotrópicas, decidimos caracterizar diferentes mutaciones pleiotrópicas. Así, podríamos valorar si existe un mecanismo común que justificase la deficiencia y sus consecuencias clínicas. Para llevar a cabo este objetivo seleccionamos mutaciones pleiotrópicas identificadas en pacientes y descritas en la bibliografía (K241E, M251I, M315K, F402L, P429L) que se localizan en diferentes hebras de la lámina C. Estas mutaciones se expresaron en un modelo recombinante. Tras su purificación, observamos que todas las mutaciones pleiotropicas, excepto M315K, generaban dos glicoformas (V1 & V2). Las variantes 1 (V1) tenían una glicosilación aberrante que difería dependiendo de la mutación. En todos los casos, la V1 presentaba una reducida afinidad por heparina y afectación severa de la reactividad con sus proteasas diana. En cambio, la variante 2 (V2) mostró una glicosilación y afinidad por heparina similares a la antitrombina control, pero con afectación de la reactividad, que era parcialmente revertida por la activación con heparina. Por lo tanto, nuestros resultados sugieren que la lámina C de la antitrombina es una región importante para la apropiada maduración de los N-glicanos, y que las mutaciones pleiotrópicas localizadas en dicha región conducen a la secreción de dos variantes, una con glicosilación aberrante (V1) dependiendo de la mutación y diferente grado de reducción de la afinidad por heparina, lo cual explicaría la heterogeneidad clínica observada en los portadores.

Objetivo 3

a) Efecto de una secuencia aromática en la glicosilación de N135.

La secuencia consenso de N-glicosilación necesaria para que actúe la OST es N-X-S/T/C. Recientemente ha sido descrito que una secuencia consenso ineficiente y localizada en un giro reverso de dos hebras β puede ser totalmente eficaz si se introduce una fenilalanina dos posiciones antes de la asparragina. Además, el anillo aromático de este residuo incrementa la estabilidad de la proteína al interaccionar con la N-acetilglucosamina del glicano. Nuestro grupo decidió evaluar el efecto de esta secuencia aromática en la N135 de antitrombina, debido a su ineficaz glicosilación; a pesar de que este sitio de glicosilación se encuentra en un contexto estructural distinto, entre una hélice y una hebra. Nuestros resultados muestran que esta secuencia (N135-K136-S137) sólo se glicosila en el 50% de las ocasiones, lo que genera la presencia de las glicoformas: α y β-antitrombina, con cuatro y tres carbohidratos, respectivamente, tal y como ha sido descrito. Al introducir la fenilalanina dos posiciones antes de la N135 la eficacia de glicosilación fue del 100%, sin provocar una mayor retención intracelular. Además, el residuo aromático afectó la afinidad por heparina y la función de la antitrombina, siendo más severa cuando la mutación K133F se combinó con la el cambio S137T. La formación de la nueva vuelta en la hélice D debido a la activación producida por la unión de heparina también se vio enlentecida o dificulatada. Sin embargo, la estabilidad global de las variantes no fue significativamente alterada, posiblemente por la elevada inestabilidad estructural de todas las serpinas. En definitiva, la fenilalanina situada dos posiciones antes de la asparragina de la secuencia consenso de N-glicosilación podría ser empleada en otro contextos estructurales distintos a un giro de dos hebras β.

b) Consecuencias funcionales de nuevos glicanos localizados en el RCL de antitrombina.

La importancia funcional de la N-glicosilación en antitrombina no solo se refleja por las diferencias funcionales derivadas de la presencia o ausencia de glicano en N135, o por las consecuencias patológicas de una glicosilación aberrante. También, mutaciones que provocan la aparición de nuevos N-glicanos se han identificado en pacientes con deficiencia de antitrombina. Las mutaciones que provocan la aparición de nuevos sitios de N-glicosilación en el HBS (I7N) o en la "región cerrojo" para la inserción del RCL (S82N), han sido identificadas en pacientes con deficiencia tipo IIb y tipo I,

respectivamente. Ya que no ha sido descrita ninguna mutación que produzca una secuencia consenso de glicosilación en el RCL de antitrombina decidimos introducir mutaciones en diferentes posiciones de este dominio funcional y evaluar su efecto funcional. Así, introdujimos dos mutaciones, una próxima y otra más alejada al residuo P1 (R393): A384N (P10) y G392N (P2). Ninguna mutación afectó a la secreción de la proteína en células HEK-EBNA, y ambas generaban formas con un nuevo glicano. Debido a que los nuevos glicanos en ambas variantes están localizados en la región de contacto con las proteasas diana, presentaban afectada su reactividad. El glicano localizado en P10 ("región bisagra"), como era de esperar, enlenteció la inserción del RCL en la lámina A central y, por lo tanto, la forma variante no formó complejos ni con FIIa ni FXa, comportándose como sustrato. Por el contrario, la variante N392 (P2), mostraba una velocidad de inhibición con FXa similar a la antitrombina control, pese a tener una estequiometria de inhibición incrementada. Sin embargo, esta variante no reaccionó con FIIa en ausencia de heparina. Este impedimento fue parcialmente eliminado por la unión de heparina, aunque la reactividad permanecía todavía fuertemente afectada. La especificad de proteasa de este último mutante podría ser muy interesante para una terapia anticoagulante más segura, ya que favorece la inhibición del FXa, al igual que las heparinas de bajo peso molecular.

Conclusiones

1. La heterozigosis compuesta que implica a la mutación A384S, es relativamente frecuente en pacientes con deficiencia de antitrombina (2/96, 2%). Este estado afecta significativamente la capacidad anticoagulante e incrementa tanto el riesgo trombótico como la severidad clínica. Estos datos junto a la ineficacia de los test trombofílicos empleados, para detectar la mutación A384S, animan a evaluar esta alteración mediante métodos moleculares en estudios familiares de pacientes con deficiencia de antitrombina, incluso si el probando no presenta esta alteración genética.

2. Identificamos una nueva región en antitrombina (la puerta de salida del loop reactivo internalizado en la lámina A central) relevante en el mecanismo inhibitorio de esta serpina, concretamente afecta a la inserción del loop reactivo durante la traslocación de la proteasa al polo opuesto de la antitrombina para su completa inhibición..

3. La lámina C es una región relevante en la correcta maduración de los N-glicanos de antitrombina. Mutaciones en esta región provocan la aparción de una variante con glicosilación aberrante que provoca la reducción de la afinidad por heparina.

4. La introducción de una secuencia aromática (Phe-X-Asn-Y-Thr) en posición 135 de la antitrombina consigue una glicosilación completamente eficaz, dificultando la activación inducida por la unión de heparina. Estos resultados apoyan que las secuencias aromáticas podrían ser empleadas para favorecer una glicosilación más eficiente en diferentes contextos estructurales.

5. Las mutaciones que generan nuevas secuencias de glicosilación en el RCL son correctamente glicosiladas y plegadas, pero el nuevo glicano interfiere con la reactividad de la serpina por diferentes mecanismos dependiendo de la localización de glicano. El mutante G392N presenta especificidad de proteasa diana, ya que puede inhibir al FXa, pero no al FIIa, lo que podría tener implicaciones terapéuticas.

APPENDIX I

Diffusion of scientific results related with the thesis

Compound heterozygosity involving Antithrombin Cambridge II (p.Ala416Ser) in antithrombin deficiency

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Dear Sirs,

Antithrombin is a key haemostatic anticoagulant serpin. More than 220 different mutations in *SERPINC1*, the gene encoding antithrombin, have been identified in patients with thrombophilia, most of them in heterozygous state (1). In fact, homozygous deficiency is not compatible with life except for few homozygous or hemizygous patients with type II deficiency, mainly antithrombin Budapest III (p.Leu131Phe, numbering system according to the HGVS) and antithrombin Toyama (p.Arg79Cys), as these mutations present a milder thrombotic risk (2, 3). Antithrombin Cambridge II (p.Ala416Ser) is another type II deficiency, and the most prevalent in normal population (0.2%, close to a low prevalent polymorphism) (4). This antithrombin variant inefficiently inhibits thrombin (5), increases the thrombin generation potential (6), and moderately increases the risk of venous and arterial thrombosis (4, 7). Importantly, this mutation is not detected when evaluating anti-factor (F)Xa activity or antithrombin antigen levels in plasma, the ordinary screening methods for antithrombin deficiency (4). The potential haemostatic and clinical relevance of underdiagnosing this defect in the general population might be significantly exacerbated among patients with antithrombin deficiency.

Functional, molecular, biochemical, and familial analysis of 93 unrelated Spanish and Portuguese patients with antithrombin deficiency, carried out as described elsewhere (8, 9), revealed two cases with functional compound heterozygosity in the *SERPINC1* gene. In both cases, the p.Ala416Ser mutation (1, 10, 11), coexisted with a novel point mutation, previously undescribed. The first patient (P1) also carried the c.1190C>T mutation, leading to the p.Ser397Leu change that affects a highly conserved residue located at β-strand 5A. P1 is a 41-year-old male, who developed an idiopathic pulmonary embolism and a deep venous thrombosis at the age of 38. Five out of seven maternal relatives with antithrombin deficiency also developed thrombotic episodes (▶Figure 1A). P1 had antithrombin antigen levels of 76% and disulfide-linked dimers in plasma (▶Figure 1A). The expression of this mutant in HEK-EBNA cells also revealed extracellular disulfide-linked dimers and moderate intracellular retention (data not shown). All these data support a conformational effect for the p.Ser397Leu mutation, which, according to recent studies (12) may contribute to explaining the severe thrombotic phenotype observed in carriers (▶Figure 1A).

The second patient (P2), a 33-year-old woman who developed deep venous thrombosis at the age of 31, also carried the c.409 -2A>T, affecting the donor splicing signal of intron 2. P2 had a type I antithrombin deficiency according to her low antigen levels (52%) (▶Figure 1B).

No other thrombophilic factor was found in the family of the either patient. Moreover, the anti-Xa activity of all carriers with antithrombin deficiency was around 50% (▶Figure 1). Antigen levels were similar in single or compound heterozygous (▶Figure 1). However, the anti-IIa activity, which was low in both patients, showed a significant reduction in compound heterozygous, reaching almost undetectable values in P2 (8%) (▶Figure 1). The normal range for activity was 70-120%.

Inherited thrombophilic analysis aims to identify genetic risk factors for thrombosis to stratify subjects according to their thrombotic risk. The final purpose of these analyses is to facilitate the clinical management of carriers: to prevent recurrence in symptomatic patients or the first event in asymptomatic carriers (13). Antithrombin deficiency is one of the strongest risk factors for venous thrombosis, although there is considerable clinical heterogeneity (2). Thus, some mutations, such as those affecting the heparin binding domain, pose a moderate risk (3). In contrast, other inactive mutants also have a gain-of-function, which, by different mechanisms, significantly reduce the anticoagulant capacity of carriers and explains their severe thrombotic profile (12, 14). A combination of prothrombotic genetic risk factors may also increase the risk of thrombosis, as demonstrated in the case of common prothrombotic polymorphisms (15). For antithrombin deficiency, the rare combination with other severe thrombophilic defects, such as protein C or protein S deficiency associated with severe thrombosis (16). Similarly, the combination of antithrombin deficiency with FV Leiden or prothrombin, which is not unusual, also increases the risk of thrombosis (17). Finally, homozygous or hemizygous patients are very rare, and their condition is always associated with antithrombin type II deficiencies (3). Compound heterozygosity might be an addi-

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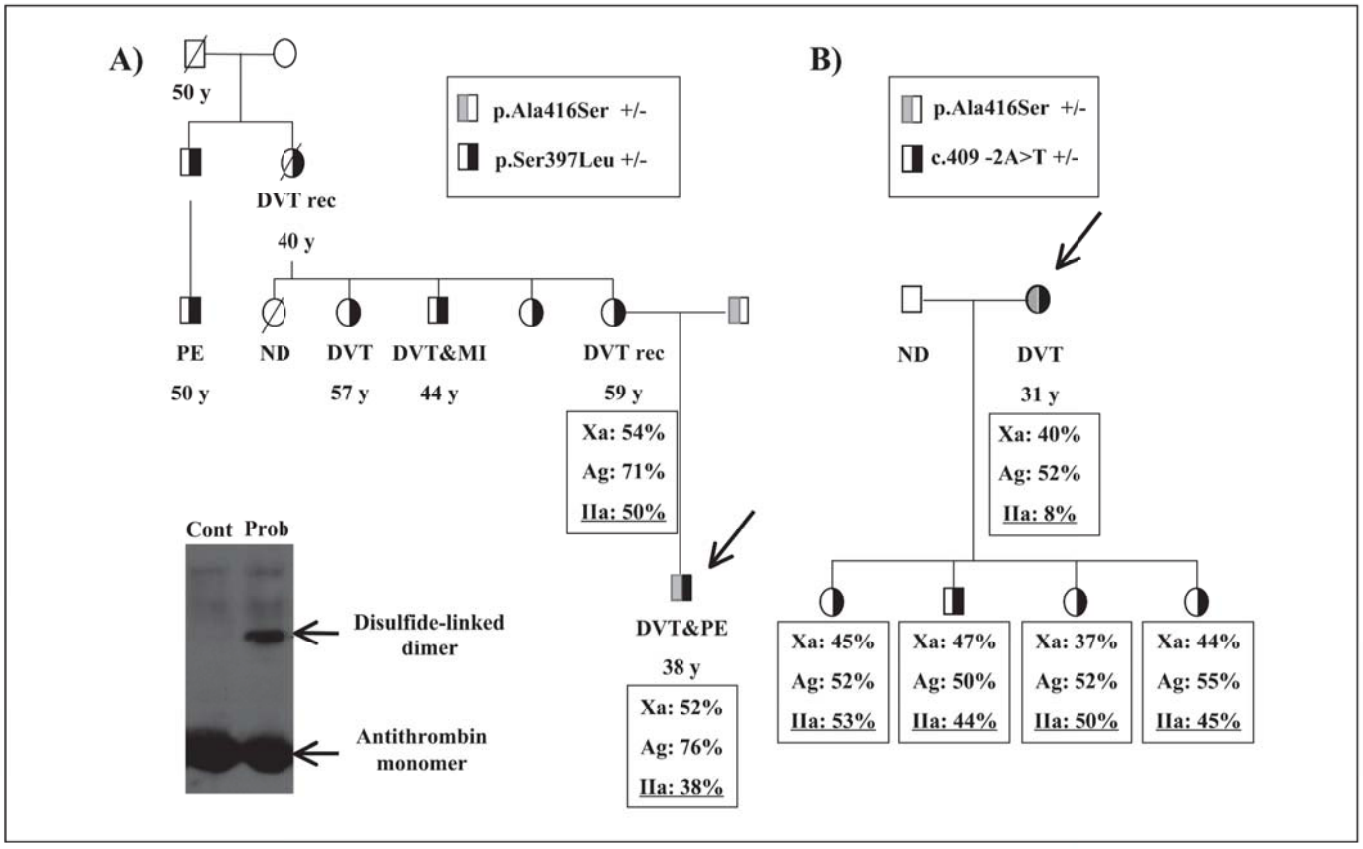

Figure 1: Clinical, laboratory and genetic data of members of families with compound heterozygosty of *SERPINC1*. A) Family 1: p.Ala416Ser & p.Ser397Leu. This figure also shows the identification by SDS under non-reducing conditions and western blot of disulfide-linked dimers of mutant antithrombin in plasma of a control (Cont) and the proband (Prob) with the S365L mutation. B) Family 2: p.Ala416Ser & c.409 -2A>T.

tional severe prothrombotic combination. Only two cases with compound heterozygosity in the *SERPINC1* gene have been described. One combines the p.Tyr198Cys mutation, which favours a failure in secretion, with a neutral p.Met52Thr mutation (18). The second was a real functional compound heterozygosity case combining an in-frame 9 bp deletion with a mild p.Arg79His mutation, leading to negligible heparin cofactor activity in compound heterozygous (19). Our study describes two cases of differing functional compound heterozygosity in the *SERPINC1* gene, but both sharing the mild p.Ala416Ser mutation. Moreover, our study suggests that this combination is relatively frequent among patients with antithrombin deficiency in Spain (2/93: 2%), where the p.Ala416Ser mutation is present in 0.2% of the normal population (4). Compound heterozygosity similar to that described here might be considered in other populations where Antithrombin Cambridge II is also a common defect in the general population (20).

The presence of functional mutations in both alleles of the *SERPINC1* gene reduced the anticoagulant activity. In addition to an impaired anti-FXa activity, which is explained by the null activity of the allele affected by the strong mutation, the anti-FIIa activity is severely reduced due to the presence of the p.Ala416Ser mutation in the other allele. These data strongly suggest that compound heterozygosity involving the antithrombin Cambridge II mutation could increase the risk or severity of thrombosis. Further studies, such as thrombin generation assays and larger family studies will be required to demonstrate this hypothesis in order to strongly support a careful clinical management of these patients, including long-term anticoagulation for symptomatic subjects and thromboprophylaxis under risk situations for asymptomatic carriers.

Finally, our results also have relevance for the accurate diagnostic of antithrombin deficiency, particularly in familial studies. Since the p.Ala416Ser is not detected by anti-FXa tests or antigen determination, and familial studies currently focused on detecting antithrombin deficiency by means of these tests or the identification of the index mutation by molecular methods, compound heterozygosity involving p.Ala416Ser might be underestimated. Actually, if the molecular analysis of the *SERPINC1* gene in family 1 had been performed in the mother of the proband (also with a severe thrombotic history) it is highly probable that the p.Ala416Ser mutation would never have been identified in the proband.

Conflicts of interest

None declared.

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ORIGINAL ARTICLE

Role of the C-sheet in the maturation of N-glycans on antithrombin: functional relevance of pleiotropic mutations

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Summary. *Background:* The characterization of natural mutants identified in patients with antithrombin deficiency has helped to identify functional domains or regions of this key anticoagulant and the mechanisms involved in the deficiency, as well as to define the clinical prognosis. Recently, we described an abnormal glycosylation in a pleiotropic mutant (K241E) that explained the impaired heparin affinity and the mild risk of thrombosis in carriers. *Objectives:* To evaluate the effects of different natural pleiotropic mutations on the glycosylation of antithrombin and their functional effects. *Methods:* Five pleiotropic mutations identified in patients with antithrombin deficiency and located at each one of the strands of the C-sheet were selected (K241E, M251I, M315K, F402L, and P429L). Recombinant mutants were generated and purified. Glycoform heterogeneity and conformational sensitivity were studied with electrophoresis, proteomic analysis, and glycomic analysis. Heparin affinity was evaluated from intrinsic fluorescence. Reactivity assays with factor Xa, thrombin and neutrophil elastase in the presence or absence of heparin were also performed. *Results and Conclusions:* Pleiotropic mutants, except for that with the M315K mutation, which affects a non-exposed residue, showed two glycoforms. Variant 1, with abnormal glycosylation, had reduced heparin affinity and severely affected reactivity with the target proteases. In contrast, variant 2, with similar electrophoretic mobility and heparin affinity to wild-type antithrombin, had impaired inhibitory activity that was partially compensated for by activation with heparin. Our results suggest the C-sheet of antithrombin as a new region that is relevant for proper maturation of the N-glycans. Therefore, pleiotropic mutations lead to glycosylation defects that are responsible for the reduced heparin affinity.

Keywords: antithrombin III; antithrombin III deficiency; glycosylation; isolation & purification; recombinant proteins.

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Introduction

Antithrombin plays a key role in the hemostatic system, as it is the main inhibitor of key procoagulant proteases such as thrombin and factor Xa. Hereditary antithrombin deficiency is a major genetic thrombotic risk factor, causing a 20-fold increased risk of venous thromboembolism [1]. More than 230 different mutations have been described in the gene coding for antithrombin (*SERPINC1*), which mostly occurs in a heterozygous state, with very few exceptions [1,2]. These deficiencies can be classified into two groups. In type I deficiency, the mutant antithrombin is not secreted to the plasma (antigen levels of $\leq 50\%$); in type II deficiency, a dysfunctional variant is detected in the plasma but it has reduced anticoagulant activity. Depending on the localization and its functional consequences, type II deficiencies are further subclassified into three groups: (i) type IIa – reactive center loop (RCL) defects, with low reactivity in the absence and presence of heparin; (ii) type IIb – heparin-binding site (HBS) defects, with impaired heparin affinity; and (iii) type IIc – multiple or pleiotropic defects caused by mutations clustered in the s1C–s4B region (Fig. S1). In types IIa and IIb, the mechanism associated with the deficiency is well defined, but in type IIc, only one study has proposed that mutations in the 402–407 region may relay structural changes to the distal HBS by perturbing the β-sheet and the core of the molecule [3].

We characterized a pleiotropic mutation identified a patient with venous thrombosis and antithrombin deficiency (K241E, antithrombin Murcia) [4]. Two variants or glycoforms were observed in the plasma of the patient carrying this mutation and in a recombinant cell system expressing the mutant protein. One glycoform had an abnormal glycosylation (core-fucosylation) that explained the reduced heparin affinity, whereas the second, with normal glycosylation and heparin affinity, had impaired inhibitory capacity that was corrected by activation with heparin [4]. The aim of this study was to determine whether other pleiotropic mutations have a similar mechanism to that described for antithrombin Murcia, and to define new potential functional regions in antithrombin.

Materials and methods

Recombinant expression of wild-type and mutant antithrombins

Mutagenesis was performed with the Stratagene QuikChange Site-Directed Mutagenesis kit (Agilent, Madrid, Spain) and the appropriate primers to obtain the K241E, M251I, M315K, F402L and P429L mutans on the pCEP4/AT-S137A plasmid that produces the β-glycoform of human antithrombin, generously provided by J. Huntington (CIMR, Cambridge, UK). Human embryonic kidney (HEK) cells expressing Epstein–Barr nuclear antigen 1 (EBNA) were grown to 60% confluence (37 °C, 5% CO_2) in Dulbecco's modified Eagle's medium with GlutaMAX-I medium (Invitrogen, Barcelona, Spain) supplemented with 5% fetal bovine serum (Sigma-Aldrich, Madrid, Spain). We transfected 200 μg mL^{-1} wild-type (WT) and mutant plasmids for 30 min in OptiMEM with LTX (Invitrogen, Life Technologies, Madrid, Spain), as suggested by the manufacturer. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and exchanged into CD-CHO medium (Invitrogen, Life Technologies) supplemented with 4 mM L-glutamine and 0.25 mg mL^{-1} Geneticin (Invitrogen). Cells were grown at 37 °C for 10 days. The medium was harvested and replaced with fresh medium every 2 days.

Secretion and intracellular retention of antithrombin variants

Cells were transfected as described previously. After 24 h of incubation with CD-CHO, the conditionated medium was withdrawn and stored at − 70 °C. Cells were then extensively washed with sterile PBS, lysed with 50 μL of lysis buffer (10 mM Tris-HCl, 0.5 mM dithiothreitol, 0.035% SDS, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate, 5 mM benzamidine, and 20 mM phenylmethylsulfonyl fluoride), and stored at − 70 °C. Intracellular and secreted antithrombins were evaluated by western blotting with 8% SDS-PAGE under reducing conditions. Antithrombin was immunostained with rabbit anti-human antithrombin polyclonal antibody (Sigma-Aldrich), followed by donkey anti-rabbit IgG–horseradish peroxidase conjugate (GE Healthcare, Barcelona, Spain), and detection was performed with an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). Monoclonal anti-β-actin (Sigma-Aldrich) was used to check sample loading.

Protein purification and electrophoretic evaluation

Recombinant proteins were purified with heparin affinity chromatography on 5-mL HiTrap Heparin columns (GE Healthcare), with an ÄKTA Purifier (GE Healthcare) in 50 mM Tris-HCl (pH 7.4) buffer; a gradient from 0 M to 3 M NaCl was applied to elute the proteins. Then, different variants of antithrombin were purified by anion exchange chromatography on 1-mL HiTrap Q columns (GE Healthcare). Those fractions containing pure antithrombin were finally desalted, concentrated, and stored at − 70 °C. The purity and separation of different variants or glycoforms were evaluated with 10% SDS-PAGE under reducing conditions and immunoblotting, as indicated elsewhere [5]. SDS-PAGE under non-reducing conditions was performed to detect disulfide-linked dimers, and urea-PAGE (6 M urea) was performed to detect latent forms [5,6].

Antithrombin activity

Antithrombin activity was determined with chromogenic anti-FXa and anti-FIIa assays in the absence and presence of heparin (HemosIL Liquid Antithrombin; Instrumentation Laboratory, Kirchheim, Germany). The protein concentration was calculated with gel densitometry and bicinchoninic acid protein assays.

Thrombin–antithrombin (TAT) complex formation

Formation of TAT complexes was evaluated by incubation of 5.4 μM antithrombin with 19 μM thrombin (Calbiochem, Millipore, Madrid, Spain) at 37 °C. Aliquots were withdrawn at different time intervals (10 min and 1 h). The reaction was carried out with and without previous incubation of antithrombin with 6.6 μM heparin for 10 min. These samples were evaluated with SDS-PAGE as indicated previously.

Interaction with neutrophil elastase

Purified glycoforms of all pleiotropic mutants were treated with 8.5 μM human neutrophil elastase (HNE) (Calbiochem) for 30 min at 37 °C in the presence of 0.4 μg of unfractionated heparin. The effectiveness of the reaction was evaluated with SDS-PAGE under non-reducing conditions, which allows the detection of cleaved antithrombin [6].

N-*glycosidase F digestion*

Purified antithrombin variants were treated with *N*-glycosidase F (Roche Diagnostics, Mannheim, Germany), which required a previous denaturing step (5 min at 100 °C in 150 mM sodium phosphate buffer, pH 7.4, and 10% NP-40). Then, samples were chilled on ice and digested with 0.6 U of *N*-glycosidase F by incubation at 37 °C overnight. Samples were resolved with 10% SDS-PAGE under reducing conditions, and detected by silver staining.

Intrinsic fluorescence studies

The equilibrium dissociation constant (K_D) for the antithrombin–heparin interaction was determined essentially as described previously [7]. Briefly, the change in the intrinsic fluorescence of antithrombin (25 nM) upon titration of the unfractionated heparin was monitored at 340 nm on a Hitachi F4500 spectrofluorometer, with excitation at 280 nm and bandwidths of 3.5 nm for both excitation and emission. All titrations were carried out at room temperature (25 °C) under physiologic ionic strength ($I = 0.15$) in 20 mM $NaPO_4$, 100 mM NaCl, 0.1 mM EDTA, and 0.1% poly(ethylene glycol) 8000 (pH 7.4). Fluorescence emission intensity was taken as the average of 100 measurements recorded at 1-s intervals for each addition of heparin. Data were fitted as previously described [7].

MALDI-TOF mass spectrometry (MS) analysis

A solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 g L^{-1}) in acetonitrile/water/trifluoroacetic acid (50 : 50 : 0.1 by volume) was used as the matrix for protein analysis. Experiments were carried out on a 4800 Plus MALDI-TOF/TOF Analyzer (ABSciex, Madrid, Sapin), equipped with a diode-pumped, solid-state laser. Recorded data were processed with DATA EXPLORER (Applied Biosystems, Life Technologies). Released carbohydrates (*N*-glycosidase F digestion; see below) were analyzed on the same instrument with 2,5-dihydroxibenzoic acid (20 g L^{-1} in methanol/water [1 : 1]) as the matrix.

Glycan profiling

Glycans were released from the antithrombin and variants with the protocol established previously [4]. Briefly, glycoprotein variants were lyophilized and resuspended in the correct deglycosylation buffer (50 mM phosphate, pH 7.3, containing 0.5% SDS). Then, 1 U of *N*-glycosidase F was added, and samples were incubated at 37 °C overnight with continuous agitation (750 r.p.m.). Glycans were separated from the deglycosylated protein by filtration over 10-kDa centrifugal filters, and subsequently purified over graphitized carbon. Following lyophilization, carbohydrates were labeled with 2-aminobenzamide (2-AB), according to the established protocol [8]. Normal-phase chromatography of 2-AB *N*-linked glycans was carried on an ACQUITY Ultra-Performance Liquid Chromatography System at Ethylene Bridged Hybrid particle of 1.7 μm (BEH UPLC) with an Agilent 1100 HPLC instrument equipped with a fluorescence detector (1100 Agilent fluorescence module, Agilent): excitation, 330 nm; and emission, 420 nm. The system was calibrated in glucose units by use of a 2-AB-labeled dextran hydrolysate. The total run time was 125 min. Samples were reanalyzed following exoglycosidase digestions, essentially α2-R sialidase and α2-R sialidase with β1–4-galactosidase, as described previously [9].

Results

Pleiotropic mutations selected

We selected pleiotropic mutations identified in patients with antithrombin deficiency from available databases (www.hgmd.org) [3,4,10–14]: K241E, M251I, M315K, F402L, and P429L. Each one of these mutations was located at one of the strands of the C sheet (s1C–s4C), except for the P429L mutation, which was located at the C-terminal extreme of antithrombin. The structural locations of these mutations are shown in Fig. S1. The pleiotropic effect of these mutations was supported by the decreased anticoagulant activity, moderately reduced antigen level, and reduced heparin affinity (Table 1).

Secretion and intracellular retention

The recombinant antithrombin mutants showed similar intracellular retention and secretion to the conditioned medium as WT antithrombin, which agrees with the classification of these mutations as provoking type II deficiency in the patients (Fig. 1A).

Purification and identification of glycoforms

Purification of the different mutants from conditioned medium was performed with heparin affinity chromatography. SDS-PAGE under reducing conditions and western blotting of eluting fractions revealed two antithrombin forms or variants (V1 and V2) with different mobilities that eluted at different salt concentrations for all mutants (data not shown), except for mutant M315K, which showed only one variant. The two variants with the same mutation were then purified and isolated by anion exchange chromatography at pH 7.4, and the purity of the different variants was evaluated with 10% SDS-PAGE under reducing conditions (Fig. S2). Unfortunately, V2 of mutant P429L was not separated completely from V1 in sufficient amounts for all assays, even with different anion exchange strategies for purification (Fig. S2).

Table 1 Clinical and laboratory data reported in the history of patients with antithrombin deficiency carrying selected pleiotropic mutations

Mutation	AT activity (%)	Ag (%)	TE	FTE (years)	Structural localization	Reference
M315K	63	78	DVT, 35 years	35	s2C	Picard *et al*. [14]
	71	95	Asymptomatic	No	NC	
F402L	56	73	rDVT	55	s1C	Lane *et al*. [11]
	55	75	rDVT	17	Conserved	
	71	73	Asymptomatic	–		Emmerich *et al*. [10]
	41	73	rDVT/PE	25		
	48	65	DVT	17		
M251I	52	73	MVT	–	s3C HC	Millar *et al*. [12]
P429L (homozygous)	36	–	Thromboembolic episodes	–	C-terminal HC	Olds *et al*. [13]
K241E	60	77	DVT	44	s4C NC	Martínez-Martínez *et al*. [4]

Ag, antigen level; AT activity, antithrombin anti-FIIa or anti-FXa activity with heparin; DVT, deep vein thrombosis; FTE, age at first thrombotic event; HC, highly conserved; MVT, mesenteric venous thrombosis; NC, not conserved; PE, pulmonary embolism; rDVT, recurrent deep vein thrombosis; TE, thrombotic event. The structural localization of the mutated residue and the conservation among the serpin superfamily are also indicated.

The form with higher heparin affinity (V2) always eluted in similar fractions and showed similar electrophoretic mobility as WT antithrombin. In contrast, V1, which eluted at low salt concentrations, showed less electrophoretic mobility than WT antithrombin. Interestingly, the mobility of V1 varied slightly according to the mutation (Fig. 1B; Fig. S2). Treatment with *N*-glycosidase F equalized the electrophoretic mobility of all variants (Fig. 1B), which suggested that differences between V1 and V2, and among V1s of different mutants, were caused by different N-glycan compositions (Fig. 1B).

MALDI-TOF MS and glycosylation analysis

The MALDI-TOF MS studies showed that the deglycosylated variants had very similar masses (~ 49 kDa), which indicates that the differences in electrophoretic mobility between V1 and V2 could be caused by different N-glycan compositions (Fig. S3).

Advanced glycomic analysis with exoglycosidase treatment and mass determination was also performed with V1 and V2 glycoforms of all pleiotropic mutants, except for mutant P429L, owing to the inefficient chromatographic separation of the two glycoforms of this variant. Unfortunately, the low amounts of purified variants did not allow us to obtain conclusive results, except for mutant M251I. Interestingly, most of the V1 glycoforms of mutant M251I contain at least one fucose residue (Fig. 2). Therefore, this is the first finding to directly identify a fucose in the abnormal glycan of a variant of antithrombin caused by a point mutation.

Heparin affinity and inhibitory activity

The heparin affinities of the different variants were evaluated with intrinsic fluorescence assays. The equilibrium dissociation constants (K_D) showed that all V1s had reduced heparin affinity as compared with those of WT antithrombin and V2s (Table 2). Thus, V1s of mutants K241E and M251I showed 4.5-fold and five-fold reductions in heparin affinity as compared with V2s and WT antithrombin, respectively, whereas the reduction was moderate for V1 of mutant F402L (three-fold) (Table 2; Fig. S4). However, there were no differences in the fluorescence increase between V1 and V2 of each of the mutants evaluated (Table 2).

The anti-FXa and anti-FIIa inhibitory activities of the different mutants were assessed with chromogenic methods, by use of the conditioned medium of HEK-EBNA cells. Both variants of all mutants showed very low inhibitory activity against both proteases in the absence of heparin (Fig. 3). Only mutant M315K (for which only one form was detected) showed slightly impaired inhibitory activity that was fully recovered by activation with heparin (Fig. 3). Furthermore, the rest of the mutants partially recovered the inhibitory activity when they were activated by heparin, excluding mutant F402L, which had negligible activity even in the presence of heparin (Fig. 3).

These results were confirmed with purified mutants by evaluation of the formation of TAT complexes at different time intervals (Fig. 4A,B). V1s of all mutants did not form covalent complexes with thrombin in the presence or absence of heparin, even after long periods of incubation (Fig. 4A). In contrast, V2s were able to form complexes with thrombin in the presence of heparin (Fig. 4B), except for V2 of mutant F402L. This mutation severely affected the reactivity of both glycoforms (Fig. 4A,B).

We also evaluated the reactivity of pleiotropic glycoforms with HNE. As shown in Fig. 5, V1s of all tested mutants did not react with HNE, as no cleaved antithrombin was detected in SDS gels under non-reducing conditions, whereas this protease cleaved V2s of the different mutants studied.

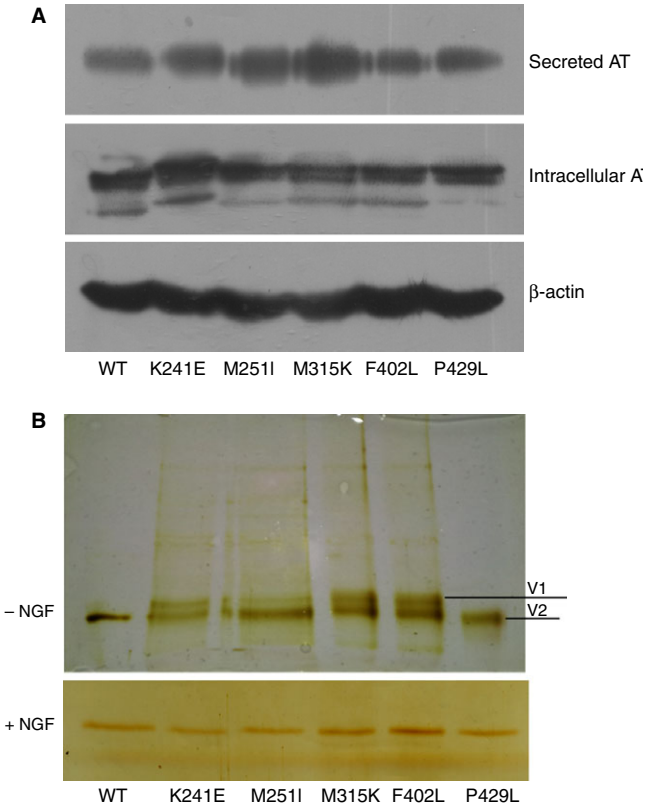

Fig. 1. Electrophoretic separation with SDS-PAGE, under reducing conditions, of recombinant antithrombins (ATs) produced and secreted by human embryonic kidney cells expressing Epstein–Barr nuclear antigen 1 and purified AT variants. (A) Western blotting of AT mutants secreted by the cells 24 h after transfection. Intracellular AT and β-actin were also evaluated. As indicated in Materials and methods, all recombinant ATs were generated in the β-context (S137A) to reduce the glycosylation heterogeneity. (B) Purified AT variants untreated with *N*-glycosidase F (– NGF) and treated with *N*-glycosidase F (+ NGF). The variants were purified and then mixed to allow easy distinction of the different mobilities. Recombinant proteins were detected by silver staining. In addition to the selected pleiotropic mutants, we also used wild-type (S137A, WT) AT as a control.

Evaluation of the conformational sensitivity of antithrombin variants

SDS-PAGE analysis under non-reducing conditions indicated that none of the variants had disulfide-linked dimers (Fig. 6A), an oligomeric form that has been reported to be associated with conformational mutations [5]. However, to check the possibility that the absence of inhibitory activity, particularly of V1s, might be attributable to non-inhibitory conformations, we evaluated the presence of hyperstable conformations with urea-PAGE. None of the mutants showed increased levels of hyperstable conformations, mainly of the latent form, in comparison with WT antithrombin (Fig. 6B).

Discussion

The characterization of natural mutations identified in patients with antithrombin deficiency has helped us to discover and characterize both functional domains of this key anticoagulant, such as the HBS, as well as to define new mechanisms involved in the deficiency of this serine protease inhibitor [4,15–19]. Moreover, these studies might also help to identify the clinical prognosis associated with each mutation [2,4]. This study was aimed at characterizing a type of antithrombin deficiency scarcely studied so far, whose underlying mechanisms are not well known and that has a wide range of clinical presentations: type IIc or pleiotropic. Thus, analysis of these mutations has allowed the identification of a new functional region in antithrombin that indirectly influences the heparin affinity and anticoagulant capacity of this protein. Moreover, our data support the relevance of an abnormal glycosylation associated with these mutations that might affect the clinical severity.

Different studies have suggested that glycosylation might play a role in both the physiology and pathology of this serpin [2,4,20–22]. In fact, antithrombin is a glycoprotein with four N-glycosylation sites (Asn96, Asn135,

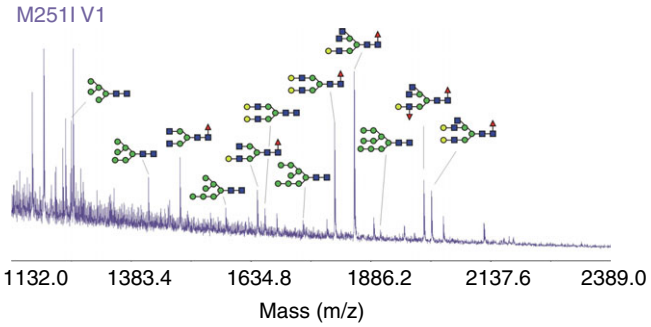

Fig. 2. Spectra of glycan composition of V1 with the M251I mutation obtained by mass spectrometry after digestion with *N*-glycosidase F. The red triangles represent fucose, the yellow circles represent mannose, the green circles represent glucose, and the blue squares represent *N*-acetylglucosamine.

Table 2 Equilibrium dissociation constants for the binding of unfractionated heparin by antithrombin (AT)

Recombinant ATs	K_D (nM)	$(\Delta F_{max}/F_o) \times 100$
WT	37.6 ± 3.1	38.3
K241E V1	257.6 ± 2.8	41.0
K241E V2	55.7 ± 4.5	42.6
M251I V1	290.0 ± 2.4	38.4
M251I V2	50.0 ± 5.3	38.7
M315K	52.9 ± 5.6	41.3
F402L V1	151.9 ± 1.5	41.6
F402L V2	50.7 ± 1.0	37.5
P429L V1 + V2	72.9 ± 4.0	41.0
P429L V2	48.2 ± 2.2	41.3

Dissociation constants (K_D) and maximal fluorescence increase were measured by global fitting of fluorescence titrations with unfractionated heparin as described in Materials and methods.

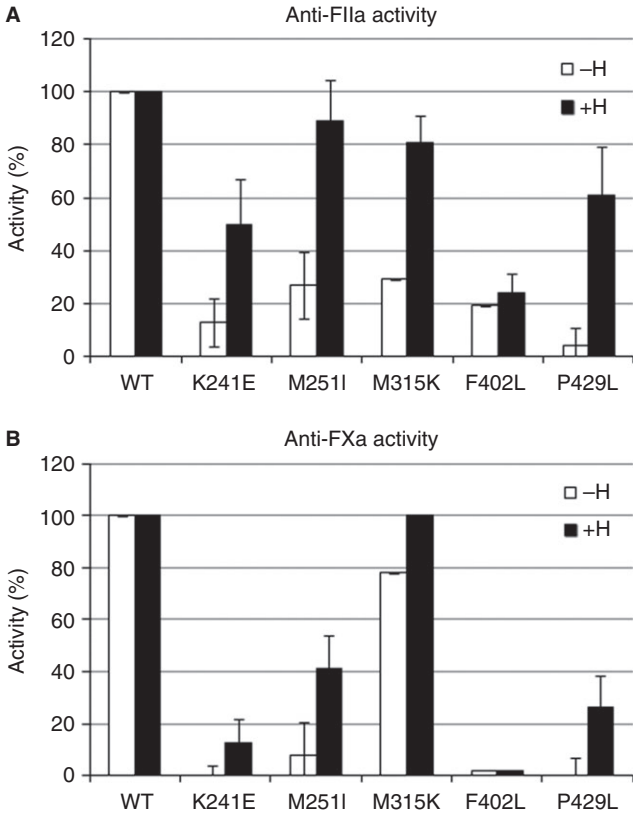

Fig. 3. Anticoagulant activity of antithrombin variants by chromogenic assays. Anti FIIa (A) and anti-FXa (B) activities of antithrombin proteins secreted to conditioned medium in the absence (gray) or presence (black) of heparin (H). Results are expressed as a percentage of the activity of wild-type (WT) antithrombin, which was used as the reference (100%). Each bar represents the mean ± standard deviation of two independent experiments performed in duplicate.

Asn155, and Asn192). The presence of the consensus sequence Asn-X-Ser instead of Asn-X-Thr at Asn135 reduces the efficiency of N-glycosylation [23,24], explaining the presence in plasma of healthy subjects of two antithrombin glycoforms, α and β, with four and three

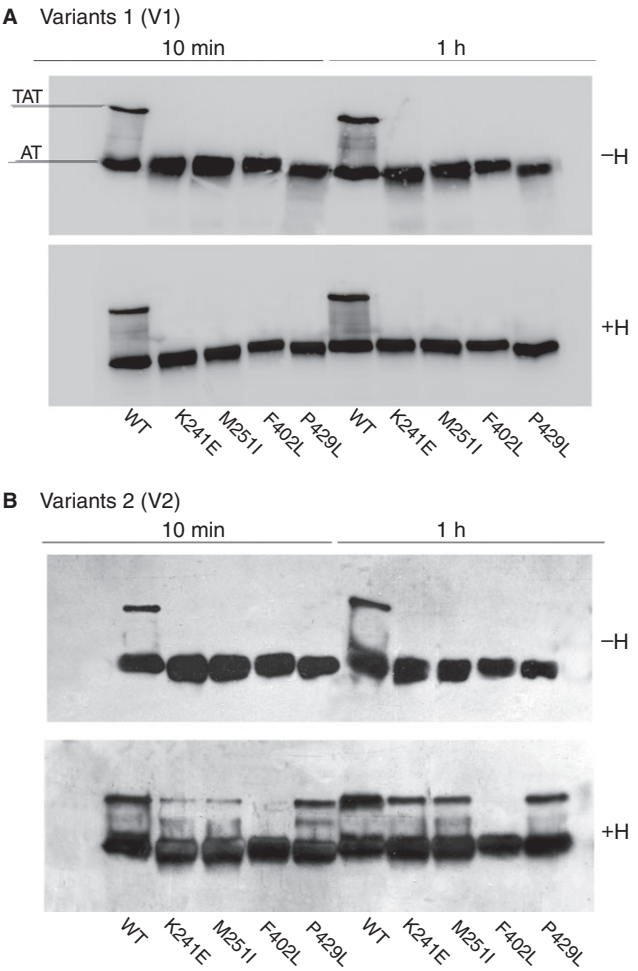

Fig. 4. Formation of thrombin-antithrombin (TAT) complexes. The wild-type (WT) antithrombin (AT) and V1 (A) and V2 (B) of each pleiotropic mutant were incubated with thrombin (10 min or 1 h) in the presence (+H) or the absence (–H) of unfractionated heparin and identified by SDS-PAGE under reducing conditions and western blotting.

N-glycans, respectively.. The lack of carbohydrate at Asn135 contributes to the higher heparin affinity of the β-glycoform and its faster clearance than those of the α-glycoform. These features also explain the minor proportion of β-antithrombin in plasma (10%) and the anticoagulant capacity of this glycoform [23,24]. Interestingly, our group has recently demonstrated that variable glycosylation modulates the effect of mutations affecting the binding of heparin [2]. Thus, the absence of a carbohydrate confers on the β-glycoform an advantage for the interaction with the heparin, as it lacks steric hindrance for the interaction. An opposite situation, fucosylation of the N-glycan at Asn155, explains the low heparin affinity of recombinant antithrombin forms expressed in baby hamster kidney cells, as this unusual glycan impairs the interaction with heparin [21]. Moreover, the fucosylation indirectly caused by the K241E mutation was also responsible for the low heparin affinity of this pleiotropic mutant [4]. In this study, we analyzed different pleiotropic

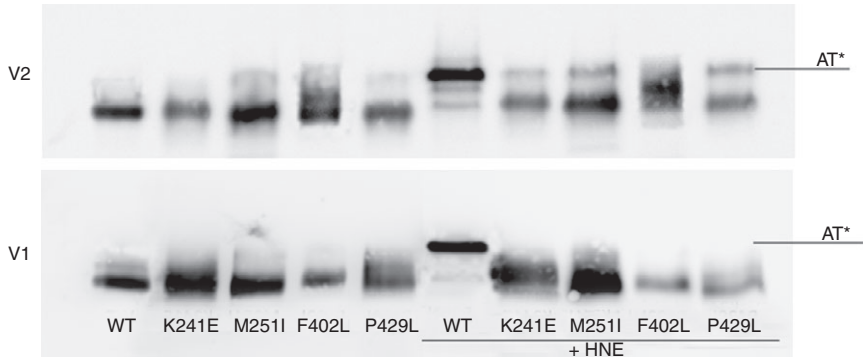

Fig. 5. Electrophoretic separation with SDS-PAGE, under non-reducing conditions, and western blotting of V1s, V2s and wild-type (WT) antithrombin (AT) after incubation with human neutrophil elastase (HNE) and unfractionated heparin for 30 min. The black lines indicate cleaved AT (AT*).

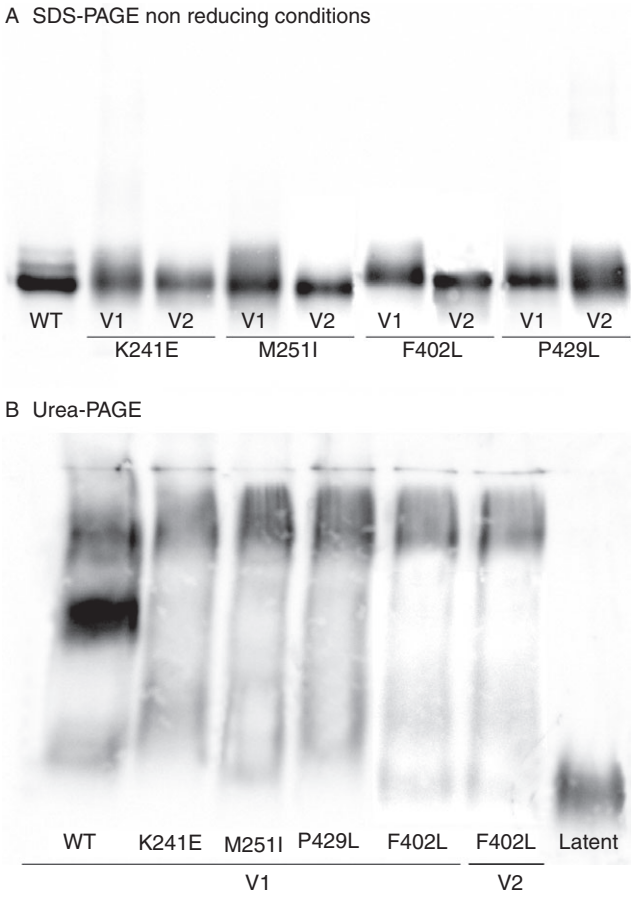

Fig. 6. Evaluation of hyperstable conformations of purified variants of pleiotropic mutants. (A) Electrophoretic separation with SDS-PAGE, under non-reducing conditions, and western blotting. (B) Electrophoretic separation with urea-PAGE and western blotting. Latent antithrombin of plasma was used as a control. Wild-type (WT) antithrombin was used as the control in both cases.

mutations affecting the C-sheet in order to determine whether there is a mechanism shared by this group of deficiencies. The analysis of these recombinant antithrombin mutants showed the presence of two glycoforms (V1 and V2). V1 showed less electrophoretic mobility in SDS gels under reducing condition than WT antithrombin and V2. These differences disappeared after *N*-glycosidase F treatment, as verified by MALDI-TOF MS analysis, indicating an abnormal composition of the carbohydrates associated with these mutations. Additionally, V1 showed different electrophoretic mobilities according to the mutation. These results suggest that each mutation might result in a different glycan composition, which could explain the heterogeneity of abnormal glycoforms associated with the different pleiotropic mutations. Moreover, the direct identification of a fucose in V1 of mutant M251I confirmed the alteration in glycosylation induced by this mutation, which explains the reduced heparin affinity of this variant, and supports the idea that the differences between V1 and V2 are caused by different glycan contents. It is important to point out that this is the first finding to directly identify a fucose in antithrombin with a missense mutation. However, further studies are required to establish the abnormal glycosylation of V1s of the other pleiotropic mutants. Collectively, these results indicate that the C-sheet might be a region involved in the maturation of N-glycosylated antithrombin. However, the mechanism leading to such abnormal N-glycan maturation is unknown. Our first study suggested that the K241E mutation, by significantly changing the electrostatic potential of the molecule, might interfere with the interactions with fucosyltransferases [4], but this hypothesis cannot be extended to the rest of the mutations tested in this study, as none of them had an effect on the electrostatic potential. Moreover, these mutants were generated in a β-context, lacking glycosylation at Asn135, which shows that the reduced heparin affinity and reactivity of V1 observed in this study must be caused by the abnormal maturation of the N-glycans of the three other glycosylation sites.

Interestingly, both V1 and V2, which share the same mutation and differ only in the N-glycan content, are not

able to interact with thrombin in the absence of heparin, even with long periods of incubation. These results support previous data suggesting that mutations in the C-sheet significantly interfere with the serpin–protease interaction [3,25–27]. However, V1 does not show inhibitory activity even in the presence of heparin, so the mutation in the C-sheet and the alteration in the glycan content still impair the interactions with the target proteases. Moreover, the defective reactivity of V1 of these pleiotropic mutants also affects the interaction with HNE, explaining the absence of inhibitory activity and cleaved forms. In contrast, binding of heparin to V2 causes correct activation of the molecule, which seems to eliminate the deleterious consequences of the C-sheet mutation on the interactions with the proteases, allowing full anticoagulant activity and the proteolytic activity of HNE, with the exception of F402L. Our data agree with those obtained by Lane *et al*., who suggested a conformational linkage between the reactive site and heparin-binding regions of the molecule in s1C variants [3]. In summary, mutations at the C-sheet have a common and relevant functional consequence: the mutation impairs the reactivity with the target proteases. However, this effect could be abolished in V2 by activation with heparin, as the glycosylation of these glycoforms seems to be similar to that of WT antithrombin. The abnormal maturation of the N-glycan combined with the mutation present on V1s of pleiotropic mutants means that they lack reactivity and the presence of heparin does not restore the effect of the mutation.

The exception to this rule is F402L, as V2 apparently had normal glycosylation and heparin affinity, but did not recover anticoagulant activity after activation with heparin. This feature might also explain the severe clinical phenotype reported for patients carrying this mutation [3,10]. As it has been demonstrated that Thr401 directly interacts with Glu37 of FXa [28], we speculate that Phe402 might be relevant for the interaction with the target proteases, and that the F402L mutation might impair the interaction between these residues. Further studies are required to verify this hypothesis.

The potential role of a conformational effect of pleiotropic mutations on the functional consequences observed in this study was ruled out. It is well known that missense mutations affecting the shutter or regions involved in the mobility of the RCL may affect the correct folding or stability of this protein, leading to inactive conformations. [5,6]. Moreover, Bottomley *et al*. found that alterations in s1C of antitrypsin could cause the serpin to become unstable [29]. Additionally, it has been reported that the lack of glycosylation in plasminogen activator inhibitor-1 changes the transition to latency in this molecule [30]. However, our results show that none of the pleiotropic mutants evaluated in our study form disulfide-linked dimers, and we did not detect increased levels of the latent form in these non-functional variants as compared with WT antithrombin. Moreover, the V1 mutants do not have altered forms (latent or polymerized), because a fluorescence increase occurs when the serpins are incubated with heparin, and this is diagnostic for a native-like conformation. Finally, the fact that V2s, when activated by heparin, have inhibitory activity and react with HNE demonstrates the presence of an exposed RCL and a native conformation. Therefore, these data suggest that pleiotropic mutations do not cause significant structural instability.

In conclusion, our study on pleiotropic mutations (K241E, M251I, F402L and P429L) identified in patients with antithrombin deficiency and thrombosis reveals that the C-sheet seems to be relevant for the correct maturation of the N-glycans. The only exception is mutant M315K, probably because the localization of this residue, close to the reactive center, might not affect the normal interaction with glycosyl transferases or glycosidases. Alternatively, these mutations might affect the folding and/or normal traffic of the protein from the endoplasmic reticulum to the *trans*-Golgi, which might allow the interaction of antithrombin with enzymes involved in N-glycosylation that, normally, hardly interact. Independently of the mechanism involved, the final result is abnormal maturation of the N-glycans of antithrombin variants with mutations in the C-sheet that impair heparin affinity and reactivity. Finally, it is important to point out that our results agree with the clinical phenotype of patients carrying these mutations, explaining the different clinical severities observed in this group of antithrombin deficiencies.

Addendum

J. Corral and I. Martínez-Martínez were the principal investigators and take primary responsibility for the paper. S. Águila, J. Navarro-Fernández, and N. Bohdan performed the laboratory work for this study. R. Gutiérrez-Gallego performed the glycomic and MS studies. I. Martíne-Martínez, J. Corral, and V. Vicente coordinated the research. I. Martínez-Martínez, S. Águila, and J. Corral wrote the paper. M. E. De la Morena-Barrio, V. Vicente, and R. Gutiérrez-Gallego critically reviewed the paper.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Ribbon diagram of native antithrombin (Protein Data Bank code 1T1F).
Fig. S2. SDS-PAGE, under reducing conditions, of purified antithrombin variants.
Fig. S3. Mass spectra obtained by MALDI-TOF MS analysis of the purified variants (V1 and V2) of pleiotropic mutants after treatment with *N*-glycosidase F.
Fig. S4. Heparin-binding titrations.

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APPENDIX II

Diffusion of scientific results unrelated with the thesis

Control of post-translational modifications in antithrombin during murine post-natal development by miR-200a. Teruel R, Martínez-Martínez I, Guerrero JA, González-Conejero R, de la Morena-Barrio ME, Salloum-Asfar S, Arroyo AB, **Águila S**, García-Barberá N, Miñano A, Vicente V, Corral J, Martínez C. J Biomed Sci. 2013. 20(1): 29.

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