UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Expanding the Continuum of Antithrombin Deficiency: New Insights on this Severe Thrombophilia via the Comprehensive Analysis of Cases in Atypical or Emerging Clinical Scenarios

Expandiendo el Continuum de la Deficiencia de Antitrombina: Nuevos Conocimientos sobre esta Trombofilia Grave por medio de la Caracterización de Casos Clínicos en Escenarios Atípicos o Emergentes

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2022

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Doctoral Dissertation

Memoria Tesis Doctoral con Mención Internacional

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New insights on this severe thrombophilia via the comprehensive analysis of cases in atypical or emerging clinical scenarios

Expandiendo el continuum de la deficiencia de antitrombina:

Nuevos conocimientos sobre esta trombofilia grave por medio de la caracterización de casos clínicos en escenarios atípicos o emergentes

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2022

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Acknowlegements

This research was conducted at Centro Regional de Hemodonación, Department of Haematology and Medical Oncology of Morales Meseguer University Hospital, University of Murcia, from September 2018 to November 2021. The study was supported by grants from Instituto de Salud Carlos III and FEDER (PI18/00598, PI21/00174, CM20/00094), and by Fundación Séneca (19 873/GERM/15).

First, I am deeply grateful to my supervisors, Dr. Maria E de la Morena-Barrio and Prof. Javier Corral. I must thank them for their continuous support during first my master studies in their laboratory, since September 2016, and later during my PhD studies, as well as for their invaluable insights and knowledge in the field of Experimental and Translational Haematology. From them I have learned the importance of paying attention to small details, and looking at singular clinical cases as a source of inspiration for biomedical research. I must also thank them for their patience, since this entire PhD period coincided first with my postgraduate training as a medical doctor in Haematology, and afterwards with my duty as a clinician when the SARS-CoV-2 pandemic emerged. And I would also like to thank them for their contagious enthusiasm, and positive attitude. It has been an absolute pleasure to work in their laboratory for 6 years.

I also manifest my most profound gratitude to my tutor, Prof. Vicente Vicente, senior haematologist and researcher, and former head of the department. He has shared with me his vast knowledge and expertise in Haematology, to which he has been totally dedicated, in all its facets: patients, research and teaching. I would like to thank him for putting me in the way of my PhD supervisors, for his wise advice, and for believing and betting on me ahead of everything, as only an extraordinary mentor does.

I would also like to thank all the external co-authors that have contributed to the research included in this thesis: JM Gomez-Verdu and Prof. MT Herranz (Servicio de Medicina Interna, Hospital Universitario Morales Meseguer, Murcia, Spain); E Bernal (Sección de Enfermedad Infecciosas, Hospital Universitario Reina Sofía, Murcia, Spain); L Entrena (Hospital Universitario Virgen de la Nieves, Granada, Spain); A Palomo (Hospital Regional Universitario

Carlos de Haya, Málaga, Spain); S Herrero (Hospital Universitario de Guadalajara, Guadalajara, Spain); S Marcellini (Hospital General de Segovia, Segovia, Spain); A Mora-Casado (Hospital Clínico San Carlos, Madrid, Spain); JM García and MS Infante (Hospital Universitario Infanta Leonor, Madrid, Spain); JJ Rodríguez-Sevilla (Hospital del Mar, Barcelona, Spain); R Gindele, P Ilonczai, M Speker, Z Oláh, A Schlammadinger and Prof. Z Bereczky (University of Debrecen, Debrecen, Hungary); and C Orlando and K Jochmans (Universitair Ziekenhuis Brussel, Brussels, Belgium).

I also would like to thank to my colleagues, also co-authors of this work, in the Department of Hematology of Morales Meseguer University Hospital and Centro Regional de Hemodonación. In alphabetical order: A Miñano, B de la Morena-Barrio, E Navarro, I Zuazu, J Padilla, ML Lozano, N Revilla, P Garrido and R Cifuentes. I specially thank Ms. Antonia Miñano for her master technical assistance in all the laboratory work performed in this thesis: brilliant, expert, and always available. We will never do Western blots like she does.

I also thank the rest of researchers from Centro Regional de Hemodonación, my other clinical colleagues at the Department of Haematology and Medical Oncology of Morales Meseguer University Hospital and Prof. Maria L Lozano, head of the department.

These acknowledgements section would not be complete if I did not explicitly thank all patients and their families, who have accepted to be part of one of the largest cohorts of antithrombin deficiency worldwide. They are the primal reason for our work in the clinic and the laboratory.

And I would finally and foremost like to express my deepest gratitude to my family, for all their love and support; from the genes they haphazardly transferred to me at my early beginning, to the education and values that they have instilled in me afterwards; and who I intend to honor at any single of my thoughts and actions.

Murcia, January 2022

Carlos Bravo Pérez

List of abbreviations

aa	Amino acids
AT	Antithrombin
AVK	Antivitamin K
bp	Nucleotide base pairs
CDG	Congenital disorder of glycosylation
CIE	Crossed immunoelectrophoresis
COVID-19	Coronavirus disease-19
CT	Computed tomography
C-term	C-terminal
Da	Dalton
DNA	Deoxyribonucleic acid
DOAC	Direct oral anticoagulant
DVT	Deep venous thrombosis
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
FDA	Food and Drug Administration.
FGG	Fibrinogen gamma chain.
FII/ FIIa	Prothrombin/ Thrombin
FV/ FVa	Factor V/ Activated factor V
FVII/ FVIIa	Factor VII/ Activated factor VII
FVIII/ FVIIIa	Factor VIII/ Activated factor VIII
FIX/ FIXa	Factor IX/Activated factor IX
FX/ FXa	Factor X/ Activated factor X
FXI/ FXIa	Factor XI/ Activated factor XI
FXII/ FXIIa	Factor XII/Activated factor XII
FXIII/ FXIIIa	Factor XIII/Activated factor XIII
GWAS	Genome-wide association study
h	Hours
HBS	Heparin binding site

HCII	Heparin cofactor II
HGMD®	Human Genetic Mutation Database®
HMWK	High molecular weight kininogen
HPLC	High performance liquid chromatography
II/HBS	Type II Heparin binding site
II/RS	Type II Reactive site
II/PE	Type II Pleiotropic effect
INDEL	Insertions/ deletions
IU	International Units
IVC	Inferior vena cava
Kal	Kalicrein
MAF	Minor allele frequency
min	Minutes
MLPA	Multiple ligation-dependent probe amplification
MPI	Mannose-6-phosphate isomerase
NGS	Next generation sequencing
N-term	N-terminal
OR	Odds ratio
PAI-1	Plasminogen activator inhibitor-1
PC	Protein C
PCI	Protein C inhibitor
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PE	Pulmonary embolism
PK	Prekalikrein
PS	Protein S
R	Relaxed conformation
RCL	Reactive centre loop
RNA	Ribonucleic acid
S	Stressed conformation

SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
SERPIN	Serine protease inhibitor
SERPINC1	Serine protease inhibitor C1
STR	Short tandem repeats
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tPA	Tissue plasminogen activator
uPA	Urokinase-type plasminogen activator
UTR	Untranslated region
VTE	Venous thromboembolism
WES	Whole exome sequencing
ZPI	Protein Z-dependent protease inhibitor

Comments on nomenclatures and symbols

- **Comment 1. Nomenclature and symbols for nucleotides and amino acids.** Terms and abbreviations used for nucleotides and amino acids commonly found in nucleic acids and proteins, are in accordance to International Union of Pure and Applied Chemistry (reference A) and standard symbolism.

Nucleotide base	Symbol
Adenine	**A**
Cytosine	**C**
Guanine	**G**
Thymine	**T**
Uracil	**U**

Amino acid	Symbol (3 and 1 letters)	
Alanine	Ala	**A**
Arginine	Arg	**R**
Asparagine	Asn	**N**
Aspartic acid	Asp	**D**
Cysteine	Cys	**C**
Glutamine	Gln	**Q**
Glutamic acid	Glu	**E**
Glycine	Gly	**G**
Histidine	His	**H**
Isoleucine	Ile	**I**
Leucine	Leu	**L**
Lysine	Lys	**K**
Methionine	Met	**M**
Phenylalanine	Phe	**F**
Proline	Pro	**P**
Serine	Ser	**S**
Threonine	Thr	**T**
Tryptophan	Trp	**W**
Tyrosine	Tyr	**Y**
Valine	Val	**V**

- **Comment 2. Numeration of nucleotides/ codons and nomenclature of variants.** The numeration of nucleotides/ codons within a gene/ protein, respectively, and the nomenclature of variants are in accordance to the standard nomenclature recommendations of the Human Genome Variation Society (http://www.HGVS.org/mutnomen/)(reference B).
- **Comment 3: All numerations include the N-terminal signal peptide.** All numerations of nucleotides/ codons are in accordance with full-encoded gene sequence, so that if a gene encodes a precursor protein with a N-terminal signal peptide, it will be included for numeration (otherwise, it would be specified).

References

A: International Union of Pure and Applied Chemistry (1979) Nomenclature of Organic Chemistry, Sections A, B, C, D, E, F and H, Pergamon Press, Oxford.

B: Horaitis O, Cotton RG. The challenge of documenting mutation across the genome: the human genome variation society approach. Hum Mutat. 2004;23:447–452.

Summary

Resumen extenso de la memoria

Introducción. La antitrombina es una serpina (inhibidor de serina proteasa) que juega un papel clave en la hemostasia. Constituye uno de los anticoagulantes endógenos más potentes. Por consiguiente, la deficiencia congénita de antitrombina es una de las trombofilias más graves y se asocia a un incremento significativo en el riesgo de enfermedad tromboembólica venosa, que se presenta de forma precoz y recurrente. La deficiencia congénita de antitrombina afecta a un 0,02-0,2% de la población general y sigue un patrón de herencia autosómico dominante, con penetrancia incompleta y expresividad variables. Se identifica por una actividad anticoagulante en plasma inferior al 80% (rango de la normalidad: 80 - 120%). El tratamiento anticoagulante se indica en aquellos individuos con deficiencia congénita de antitrombina que desarrollan un evento tromboembólico. Además, la tromboprofilaxis primaria está indicada en situaciones de alto riesgo trombótico, tales como el embarazo y la cirugía. Existen concentrados de antitrombina derivados de plasma y preparados recombinantes.

Hasta la fecha, se han descrito más de 400 mutaciones patogénicas en SERPINC1, el gen codificante de la antitrombina, que provocan dos grandes tipos de deficiencia: tipo I (cuantitativa) y tipo II (cualitativa). Las deficiencias de antitrombina tipo I se corresponden con un defecto en la producción o secreción proteica. Habitualmente, son causadas por mutaciones con pérdida de sentido o pequeñas deleciones/ inserciones en heterocigosis, ya que en homocigosis son letales. La mayoría de estos cambios genéticos alteran la síntesis proteica, pero en otras ocasiones afectan al procesamiento post-traduccional, al plegamiento o a la estabilidad de la proteína. Por su parte, las deficiencias tipo II se relacionan con variantes aberrantes de antitrombina, que se producen y se secretan, pero tienen reducida o nula actividad anticoagulante. Se han descrito tres subtipos de mutaciones tipo II, dependiendo de la localización de la mutación y el defecto funcional: reactivas (II/RS), principalmente afectando al centro reactivo, de sitio de unión a heparina (II/HBS) y de efecto pleiotrópico (II/PE).

Cincuenta años desde que Egeberg describiera en 1965 la primera familia trombofílica con deficiencia de antitrombina, la identificación y estudio de las variantes de antitrombina ha permitido profundizar en el conocimiento en esta serpina, que se ha trasladado al conjunto de inhibidores de serina proteasas. A pesar de ello, sigue existiendo una miríada de asuntos sin resolver relacionados con este apasionante anticoagulante.

Objetivos. El objetivo principal de esta tesis fue descubrir nuevos mecanismos e implicaciones de la deficiencia de antitrombina, con el fin de mejorar el diagnóstico y manejo de este trastorno. Abordamos este objetivo con un enfoque original, la caracterización de casos con deficiencia de antitrombina y manifestaciones atípicas, no asociadas previamente a esta enfermedad, o problemas de salud emergentes, como la infección por SARS-CoV-2, en la que el impacto de las trombofilias hereditarias no ha sido estudiado.

Esta tesis tuvo cuatro objetivos o líneas de investigación específicos:

1. Identificar y caracterizar casos con deficiencia transitoria de antitrombina.
2. Evaluar la prevalencia de defectos del sistema de la vena cava inferior en portadores homocigotos de la variante antitrombina Budapest 3 (p.Leu131Phe).
3. Caracterizar una familia trombofílica con deficiencia de antitrombina y un fuerte historial de muertes embriofetales y neonatales.
4. Analizar la relación entre las trombofilias hereditarias y las manifestaciones y gravedad de la infección por SARS-CoV-2.

Materiales y métodos. El punto de partida de esta tesis es una cohorte de casos no relacionados con deficiencia de antitrombina, reclutados en nuestro centro desde distintos hospitales españoles y europeos durante un periodo de 23 años (1998-2021, N = 444). Cada caso disponía de al menos dos determinaciones distintas de actividad de antitrombina, una realizada en el hospital de origen y una segunda realizada en nuestro centro (muestra de validación). En todas las líneas de investigación, el estudio molecular de los casos se sirvió de una metodología común. La antitrombina plasmática se caracterizó mediante métodos funcionales (sustratos cromogénicos) y bioquímicos (western blot). El análisis del gen SERPINC1 incluyó secuenciación por Sanger y secuenciación de nueva generación y amplificación de sondas dependiente de ligandos múltiples. Los defectos de N-glicosilación también han sido implicados en la deficiencia de antitrombina, por lo que el estado de glicosilación de la antitrombina y otras glicoproteínas plasmáticas (α1-antitripsina, transferrina) se analizó mediante western blot y cromatografía líquida de alta eficacia.

Los criterios de selección y el diseño específico de cada línea de investigación fueron los siguientes:

1. **Identificación y caracterización de deficiencias transitorias de antitrombina.** Se definieron los conceptos de deficiencia de antitrombina constitutiva, para aquellos casos que presentaban niveles de actividad anticoagulante consistentemente inferiores al 80% en todas las determinaciones, y

deficiencia transitoria, para los casos que presentaron al menos una determinación normal (≥80%). Independientemente del tipo de deficiencia, ambos grupos fueron estudiados a nivel molecular con la misma metodología, mencionada previamente.

2. **Estudio de la frecuencia de defectos del sistema de la vena cava inferior en portadores homocigotos de la variante antitrombina Budapest 3.** Se realizó un estudio transversal en pacientes homocigotos para la variante antitrombina Budapest 3, identificados en una gran cohorte multicéntrica de sujetos con deficiencia de antitrombina (N = 1118). El sistema de la vena cava inferior fue evaluado en estos casos mediante análisis de imagen, incluyendo tomografía computarizada y flebografía.
3. **Caracterización de una familia trombofílica con deficiencia de antitrombina y un fuerte historial de muertes embriofetales y neonatales.** En este caso, el estudio molecular incluyó también el análisis de tejido donado de uno de los abortos espontáneos acontecidos en esta familia.
4. **Análisis de la relación entre las trombofilias hereditarias y las manifestaciones y gravedad de la infección por SARS-CoV-2.** Se analizaron los perfiles clínicos y biológicos de pacientes con trombofilia que padecían infección por SARS-CoV-2. Se siguieron dos diseños para identificar a estos pacientes: 1) Análisis retrospectivo de la infección por SARS-CoV-2 en pacientes con diagnóstico previo de deficiencia congénita de antitrombina, procedentes de nuestra cohorte (N = 331). 2) Secuenciación completa del exoma de pacientes menores de 75 años hospitalizados por neumonía por SARS-CoV-2 (N = 87).

Resultados. A continuación, se resumen los resultados de cada línea de investigación:

1. Identificación y caracterización de deficiencias transitorias de antitrombina. Se identificaron 305 casos con deficiencia de antitrombina constitutiva, de los cuales 260 (85,2%) presentaron alteraciones moleculares: 250 presentaban alteraciones en SERPINC1 y 10 se explicaron por defectos de N-glicosilación. En los 139 casos restantes, la deficiencia de antitrombina fue transitoria. Entre los casos con deficiencia transitoria 61 eran portadores de mutaciones patogénicas (43,9%): 48 con alteraciones en SERPINC1, dos de ellas recurrentes (antitrombina Cambridge II [p.Ala416Ser] N=15, y antitrombina Dublín [p.Val30Glu] N=12), y 13 presentaban defectos de N-glicosilación. Dos causas justificaron la deficiencia transitoria de antitrombina: 1) la limitación técnica de los ensayos funcionales y 2) la combinación de una variante genética con un factor adquirido (estrés celular, generación de trombina,

abuso de alcohol), exacerbando su efecto patogénico sobre la función, conformación y/ o glicosilación de la antitrombina.

2. Estudio de la frecuencia de defectos del sistema de la vena cava inferior en portadores homocigotos de la variante antitrombina Budapest 3. Se identificaron 61 pacientes homocigotos para antitrombina Budapest 3. Se observó atresia del sistema de la vena cava inferior en 17 de 24 casos (70,8%) con estudio de imagen disponible: 16 tenían ausencia de vena cava inferior infrarrenal y uno tenía atresia de la vena ilíaca común izquierda. Todos los casos con defectos vasculares tenían mecanismos compensatorios, y siete también tenían otras anomalías congénitas. El análisis de microsatélites o marcadores cortos de repetición en tándem apoyó la asociación específica de la malformación vascular con la variante en SERPINC1.

3. Caracterización de una familia trombofílica con deficiencia de antitrombina y un fuerte historial de muertes embriofetales y neonatales. La caracterización molecular reveló dos mutaciones diferentes que coexistían dentro de la familia: una variante intrónica (c.1154-14G> A), portada por el probando, que causó una deficiencia grave tipo I; y una variante de Budapest 3 (deficiencia tipo II/HBS), portada en heterocigosis por la segunda pareja del probando. La caracterización de esta familia reveló un gradiente genotipo-fenotipo que cubría, y perfectamente ilustraba, todo el espectro clínico asociado con la deficiencia de antitrombina. Además, la caracterización molecular del tejido del aborto reveló una heterocigosis compuesta para las variantes tipo I y tipo II/HBS, lo que pudo haber llevado a una deficiencia de antitrombina extremadamente grave y letalidad embrionaria.

4. Análisis de la relación entre las trombofilias hereditarias y las manifestaciones y gravedad de la infección por SARS-CoV-2. Globalmente, combinando las dos estrategias de reclutamiento, se identificaron 15 de pacientes con trombofilia e infección por SARS-CoV-2: 4 pacientes portadores de polimorfismos protrombóticos (factor V Leiden, N = 2; protrombina G20210A, N = 2) y 11 pacientes con variantes que afectaban a los genes de la antitrombina (N = 6), la proteína S (N = 3) y proteína C (N = 2). Se encontraron niveles aumentados de dímero D en pacientes con trombofilia grave e infección por SARS-CoV-2 en comparación con pacientes sin trombofilia. Los pacientes con trombofilia que habían sufrido episodios trombóticos previos (N = 5), todos anticoagulados en el momento de la infección, no desarrollaron una trombosis sintomática durante la infección. Por el contrario, un paciente con deficiencia de proteína C identificada a raíz del estudio molecular, sin antecedentes trombóticos, presentó un

tromboembolismo pulmonar dos días después del ingreso hospitalario a pesar de la profilaxis antitrombótica con heparina.

Conclusiones. Este trabajo muestra resultados relevantes en sus todas sus líneas de desarrollo:

1. La deficiencia de antitrombina es un trastorno probablemente subestimado, ya que en nuestra cohorte hemos identificado casos con deficiencia de antitrombina transitoria. Una proporción significativa de casos con deficiencia transitoria de antitrombina presentaron base molecular: variantes en SERPINC1 y defectos de N-glicosilación. Nuestro estudio sugiere realizar un estudio molecular de la deficiencia de antitrombina en casos seleccionados que alternen valores normales y patológicos por métodos funcionales. Dos razones justifican la deficiencia transitoria: la limitación técnica de los ensayos funcionales, que apoya un estudio molecular de la deficiencia de antitrombina; y la combinación de una variante genética con un factor adquirido, exacerbando su efecto patogénico sobre la función, conformación y/ o glicosilación de la antitrombina. Estos hallazgos cambian el paradigma de la trombofilia congénita, ya que el efecto patogénico de una mutación puede depender de factores externos, así como estar presente sólo en ciertos momentos.

2. Los portadores homocigotos de la variante de antitrombina Budapest 3 tienen una alta prevalencia de atresia del sistema de la vena cava inferior. Esta es la primera evidencia sobre la asociación de una alteración genética y una trombofilia grave con esta malformación vascular, y apoya que una trombosis en los vasos fetales en desarrollo puede ser el mecanismo patogénico subyacente. Asimismo, la hipercoagulabilidad conferida por el estado trombofílico podría contribuir, junto a factores hemorreológicos, al alto riesgo de trombosis venosa observado en pacientes con atresia de la vena cava inferior durante la vida posnatal. Los resultados impulsan a realizar más estudios para investigar este y otros estados trombofílicos graves en pacientes diagnosticados de atresia de la vena cava inferior.

3. Proporcionamos nueva evidencia en humanos de que una deficiencia grave de antitrombina podría causar letalidad embrionaria, así como del papel de la antitrombina en el desarrollo embrionario.

4. Presentamos el primer estudio que aborda el papel de la trombofilia congénita en la infección por SARS-CoV-2, y que sugiere que las trombofilias hereditarias acentúan aún más el estado protrombótico de estos pacientes. Esto confirma el beneficio de la terapia anticoagulante en este desorden. Es necesario realizar más investigaciones para aclarar las consecuencias biológicas y clínicas de los estados trombofílicos en la infección por SARS-CoV-2, así como para orientar el enfoque óptimo para manejar la anticoagulación en estos casos.

En resumen, nuestro trabajo de investigación traslacional, enfocado en la deficiencia de antitrombina en escenarios clínicos atípicos o emergentes, brinda conceptos rompedores que confirman que, más de 50 años después, nos encontramos lejos de comprender completamente la antitrombina y su deficiencia. Se siguen demostrando nuevas funciones para esta enigmática glicoproteína, y no sólo en la hemostasia. Nuestro trabajo demuestra que la deficiencia de antitrombina está subestimada y podría ser incluso más heterogénea de lo esperado. Hemos ampliado el espectro clínico y molecular de la deficiencia de antitrombina: desde el comportamiento subrepticio de las deficiencias transitorias, hasta las malformaciones vasculares y la muerte embrionaria en los casos más extremos con defectos compuestos en SERPINC1. Se han identificado nuevas formas y mecanismos de deficiencia de antitrombina, que involucran otros loci genéticos en lugar de SERPINC1, tales como los que participan en las vías de N-glicosilación. Finalmente, la infección por SARS-CoV-2 se ha confirmado como un desencadenante protrombótico emergente para el cual los pacientes con esta y otras trombofilias hereditarias pueden ser una población de alto riesgo. En conjunto, este trabajo destaca la importancia del análisis molecular de la deficiencia de antitrombina, no sólo para una mejor comprensión de la biología de la trombosis, sino también en términos de implicaciones clínicas.

Brief summary

Introduction. Antithrombin is an anticoagulant serpin that plays a key role in haemostasis. Antithrombin deficiency (activity<80%) is a severe thrombophilic state. More than 400 variants have been described in SERPINC1, the gene encoding antithrombin. Characterization of many of these variants has generated plenty of knowledge.

Aims. To decipher new mechanisms involved in antithrombin deficiency by the characterization of cases in atypical/emergent scenarios, with four specific lines of research:

1. To identify and characterize cases with transient antithrombin deficiency.
2. To assess the frequency of inferior vena cava defects in patients homozygous for antithrombin Budapest 3 (p.Leu131Phe).
3. To characterize a family with antithrombin deficiency and a strong history of embryo-foetal deaths.
4. To analyse the association between inherited thrombophilias and COVID-19 complications.

Methods. Study of a cohort of 444 unrelated cases with antithrombin deficiency, recruited from 1998 to 2021. Plasma antithrombin was characterized by functional and biochemical methods. SERPINC1 was studied by Sanger's or

Resumen breve

Introducción. La antitrombina es un anticoagulante que juega un papel clave en la hemostasia. La deficiencia de antitrombina (actividad<80%) es un estado trombofílico grave. Se han descrito más de 400 variantes en SERPINC1, el gen que codifica la antitrombina. La caracterización de muchas de estas variantes ha generado un vasto conocimiento.

Objetivos. Descifrar nuevos mecanismos implicados en deficiencia de antitrombina mediante la caracterización de casos en escenarios atípicos/emergentes, con cuatro líneas:

1. Identificar y caracterizar casos con deficiencia de antitrombina transitoria.

2. Evaluar la frecuencia de defectos del sistema de la vena cava inferior en pacientes homocigotos para la variante antitrombina Budapest3 (p.Leu131Phe).

3. Caracterizar una familia con deficiencia de antitrombina y un fuerte historial de muertes embriofetales.

4. Analizar la asociación entre trombofilias hereditarias y complicaciones de la COVID-19.

Métodos. Estudio de una cohorte de 444 casos con deficiencia de antitrombina, 1998-2021. La antitrombina se caracterizó por métodos funcionales y bioquímicos. SERPINC1 se estudió mediante secuenciación y amplificación de sondas

next generation sequencing and multiplex ligation-dependent probe amplification. In line of research 1, we defined cases with transient antithrombin deficiency as those that alternated normal and defective functional values of antithrombin. In line 2, we selected cases homozygous for antithrombin Budapest 3, from 1118 patients (index cases and relatives) from two different cohorts (Spain, N=692; Hungary, N=426) for abdominal image exam. In line 3, we focused on a family with antithrombin deficiency and a strong history of embryo-foetal deaths. In line 4, two designs were followed to identify cases with thrombophilia and SARS-CoV-2 infection: 1) retrospective analysis of SARS-CoV-2 infection in subjects with prior diagnosis of antithrombin deficiency (N=331), and 2) whole exome sequencing of patients <75 years old hospitalized with SARS-CoV-2 pneumonia (N=87).

Results. The results of each line of research are summarized:

1. A total of 139 cases from our cohort presented transient antithrombin deficiency, and 61 (43.9%) of them had molecular defects: 48 had SERPINC1 variants, and 13 had hypoglycosylation. Two main mechanisms explained transient deficiency: the limitation of current functional methods to detect some variants and the influence of external factors on

dependiente de ligandos múltiples. En la línea de investigación 1, definimos casos con deficiencia transitoria de antitrombina aquellos que alternaban valores funcionales normales y defectuosos. En la línea 2, seleccionamos casos homocigotos para antitrombina Budapest3, de entre 1118 pacientes (casos índice y familiares) procedentes de dos cohortes (España, N=692; Hungría, N=426). En la línea 3, nos centramos en una familia con deficiencia de antitrombina y un fuerte historial de muertes embriofetales. En la línea 4 se siguieron dos diseños para identificar casos con trombofilia e infección por SARS-CoV-2: 1) búsqueda de casos con infección por SARS-CoV-2 en sujetos con diagnóstico previo de deficiencia de antitrombina (N=331), y 2) exoma completo de pacientes <75 años hospitalizados con neumonía SARS-CoV-2 (N=87).

Resultados. Resultados de cada línea de investigación:

1. Un total de 139 casos presentaron deficiencia transitoria de antitrombina, y 61 (43,9%) tenían defectos moleculares: 48 con variantes en SERPINC1 y 13 con hipoglicosilación. Dos mecanismos principales explicaron la deficiencia transitoria: la limitación de los métodos funcionales para detectar algunas variantes y la influencia de factores externos en las

the pathogenic consequences of these mutations.

2. Twenty-four of 61 homozygous Budapest 3 subjects recruited had an available image exam. Atresia of the inferior vena cava system was observed in 17 (70.8%) of these cases. Short tandem repeat analysis supported the specific association of the vascular malformation with the SERPINC1 variant.

3. Two different mutations were identified in this family: a severe quantitative variant (c.1154-14G>A), and antithrombin Budapest 3. Molecular characterization of a dead embryo revealed a compound heterozygosis for the variants, which may have led to extremely severe antithrombin deficiency and embryonic lethality.

4. Fifteen patients with thrombophilia and SARS-CoV-2 infection were identified. Increased at-admission levels of D-dimer were found in patients with severe thrombophilia. Patients with prior thrombotic history (N=5), all on anticoagulant therapy, did not develop symptomatic trombosis during infection. In contrast, one newly identified protein C-deficient patient, without thrombotic history, had a pulmonary embolism two days after hospital admission.

Conclusions. Our work demonstrated that antithrombin deficiency is underestimated. We have expanded the clinical and molecular continuum of antithrombin deficiency: from the

consecuencias patogénicas de estas mutaciones.

2. Veinticuatro de 61 sujetos homocigotos Budapest3 presentaban una prueba de imagen disponible. Se observó atresia del sistema de la vena cava inferior en 17 (70,8%) de estos casos. El análisis de microsatélites apoyó la asociación específica de esta malformación vascular con la variante en SERPINC1.

3. Se identificaron dos mutaciones diferentes en esta familia: una variante cuantitativa grave (c.1154-14G>A) y antitrombina Budapest3. La caracterización molecular de un embrión muerto reveló una heterocigosis compuesta para las variantes, lo que pudo provocar letalidad embrionaria.

4. Se identificaron 15 pacientes con trombofilia e infección SARS-CoV-2. Se encontraron niveles elevados de dímero D al ingreso en pacientes con trombofilia grave. Los pacientes con antecedentes trombóticos previos (N=5), todos anticoagulados, no desarrollaron trombosis sintomática durante la infección. Un paciente con deficiencia de proteína C recién identificado, sin antecedentes trombóticos, presentó una embolia pulmonar dos días después del ingreso hospitalario.

Conclusiones. La deficiencia de antitrombina es una enfermedad infraestimada. Hemos ampliado el continuo clínico y molecular de la deficiencia de antitrombina: desde las deficiencias transitorias

surreptitious behaviour of transient deficiencies to vascular malformations and embryonic death in the most extreme cases with compound SERPINC1 defects. Novel forms and mechanisms of antithrombin deficiency have been identified. Finally, SARS-CoV-2 infection has been confirmed as an emergent prothrombotic trigger for which patients with this and other inherited thrombophilias may be a high-risk population. Altogether, this work highlights the importance of the molecular analysis of antithrombin deficiency, not only for a better understanding of thrombosis biology, but also in terms of clinical implications.

hasta las malformaciones vasculares y la letalidad embrionaria. Se han identificado nuevas formas y mecanismos de deficiencia de antitrombina. Finalmente, la infección por SARS-CoV-2 se ha confirmado como un desencadenante protrombótico emergente; pacientes con esta y otras trombofilias pueden ser una población de alto riesgo. En conjunto, este trabajo destaca la importancia del análisis molecular de la deficiencia de antitrombina, no solo para una mejor comprensión de la biología de la trombosis, sino también en términos de implicaciones clínicas.

Outline of the thesis

Outline of the thesis

Over millions of years of evolution, the haemostatic system has been developed as a complex network of elements that might be divided into four main biological processes: primary haemostasis (vasoconstriction and platelet plug formation), secondary haemostasis (coagulation cascade), natural anticoagulation and fibrinolysis (Figure 1). The components of these systems are extremely interconnected, and the lack of balance between them may be the source of both thrombotic and haemorrhagic disorders.[1]

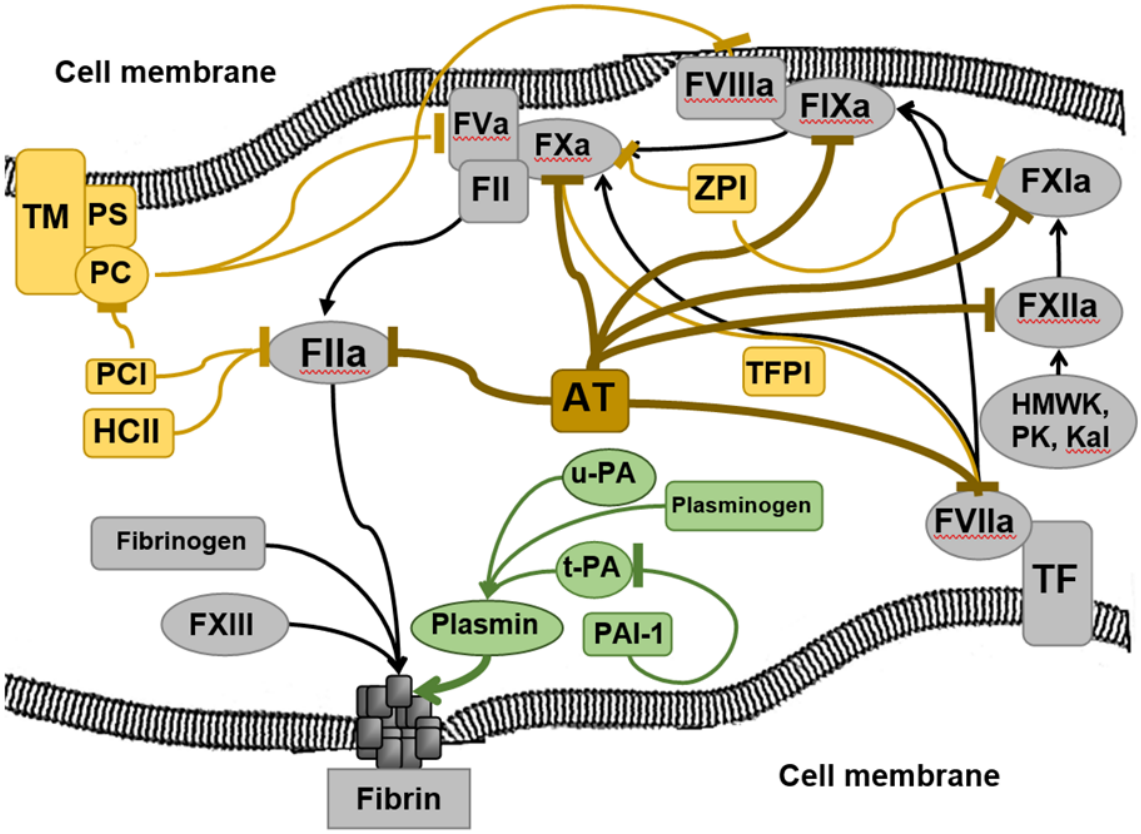

Figure 1. Coagulation factors, natural anticoagulants and fibrinolytic system. Soluble-phase elements of the coagulation cascade (grey), natural anticoagulants (yellow) and fibrinolytic (green) systems are shown. Activating and inhibiting interactions are represented as arrows and blunt ends, respectively. Antithrombin and its inhibitory targets are highlighted in dark yellow.

AT: antithrombin. FII/FIIa: prothrombin/ thrombin. FVa, VIIa, VIIIa, FIXa, FXa, FXIa, FXIIa: activated factors V, VII, VIII, FIX, FX, FXI, FXII. FXIII: factor FXIII. HCII: heparin cofactor II. HMWK: high molecular weight kininogen. Kal: kalicrein. PAI-1: plasminogen activator inhibitor-1. PC: protein C. PCI: protein C inhibitor. PK: prekalikrein. PS: protein S. TF: tissue factor. TFPI: tissue factor pathway inhibitor. TM: thrombomodulin. tPA: tissue plasminogen activator. uPA: urokinase-type plasminogen activator. ZPI: protein Z-dependent protease inhibitor.

Thrombosis is a worldwide major cause of morbidity and mortality;[2] it is a is a complex disease that arises from the combination of genetic and environmental factors.[3] The term congenital thrombophilia encompasses all genetic risk factor for thrombosis. According to the relative and absolute risk of thrombosis estimated from cohort studies, inherited thrombophilic states are divided into two groups: major and minor thrombophilias.[4] Major thrombophilia is the high-risk category, constituted by genetic diseases that predispose to early and recurrent thrombosis due to the deficiency of any of the plasma inhibitors antithrombin, protein C or protein S.[4] Minor thrombophilia is the low-risk group, and it consists of prothrombotic polymorphisms that cause mild to moderate increase in the thrombotic risk. Numerous minor thrombophilic defects have been described, but the most representative examples by far are factor V Leiden (rs6025) and prothrombin G20210A (rs1799963).[4]

Antithrombin is one of the most potent endogenous anticoagulants, since it inhibits multiple coagulation serine-proteases, mainly thrombin (FIIa) and activated factor X (FXa), but also FVIIa, FIXa, FXIa and FXIIa (Figure 1).[5–7] Consequently, congenital antithrombin deficiency significantly increases the risk of thromboembolism, and it constitutes a major thrombophilic state, the first discovered and most severe so far (Figure 2).[5]

Antithrombin deficiency was reported by Egeberg in 1965,[8] it allowed us to uncover inherited prothrombotic disorders, and has been deeply studied since then. However, more than 50 years later, antithrombin continues showing new functions, not only in haemostasis, but also in angiogenesis, inflammation and cancer.[9] Novel pathogenic mechanisms causing antithrombin deficiency have been identified, involving other genetic loci rather than SERPINC1, such as those participating in glycosylation pathways and, probably, other post-translational modifications.[9]

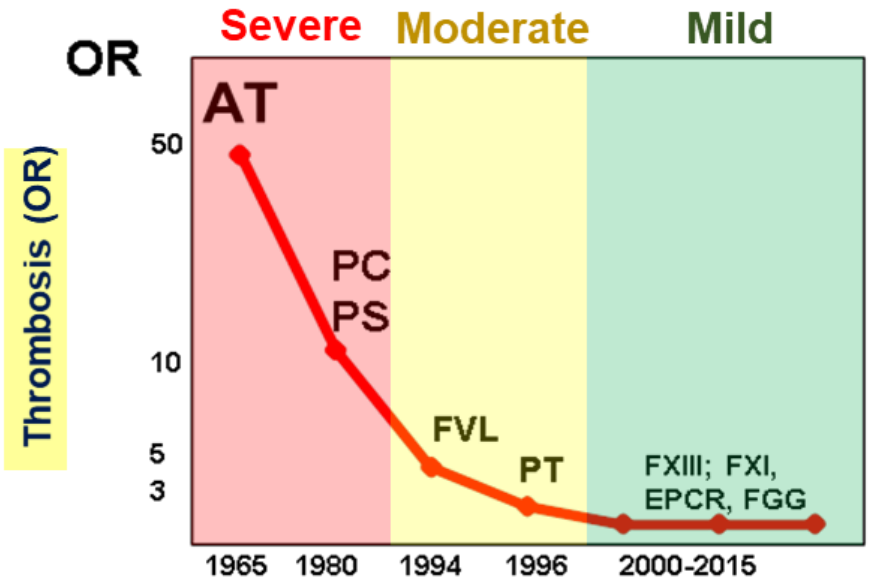

Figure 2. Main thrombophilic states. Graphic representation of major (AT deficiency, PC/PS deficiency) and minor (FVL, PT) states according to the risk of thrombosis (OR for venous thrombosis compared with healthy controls) and year of first report. Modified from: Blood. 2009: 113:5038-39.[4,10]
AT: antithrombin. EPCR: endothelial protein C receptor. FGG: fibrinogen gamma chain gene. FVL: factor V Leiden (rs6025). FXI, FXIII: factors XI, XIII: OR: odds ratio. PC: protein C. PS: protein S. PT: F2 c.*97G>A (rs1799963).

Within this thesis, we aimed to decipher new mechanisms and roles of antithrombin deficiency. We addressed this objective with an original approach, the characterization of cases with antithrombin deficiency and atypical manifestations, those with unusual laboratory or clinical findings, not previously associated to this prothrombotic disease, or with emergent health problems, such as Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infection and coronavirus disease-19 (COVID-19), in which the impact of inherited thrombophilias has not been studied. Our premise was that looking at a problem in a different light may help to solve some of its riddles.

This thesis has been written as a compendium of publications and can be divided into seven chapters:

Chapter 1 is the General introduction.

Chapter 2 is the Aims and lines of research section.

Chapter 3 is the Summary of publications.

Chapter 4 is the General discussion.

Chapter 5 is the Conclusions section.

Chapter 6 is the References section.

Chapter 7 is the Compendium of publications of this thesis, which is composed by six papers:

- Publication 1: Carlos Bravo-Pérez, Vicente Vicente, Javier Corral. Management of antithrombin deficiency: an update for clinicians. Expert Rev Hematol. 2019;12(6):397-405. doi: 10.1080/17474086.2019.1611424. Received 19 Feb 2019, Accepted 23 Apr 2019.
- Publication 2: Carlos Bravo-Pérez, Maria E de la Morena-Barrio #, Vicente Vicente #, Javier Corral #. Antithrombin deficiency as a still underdiagnosed thrombophilia: a primer for internists. Pol Arch Intern Med. 2020;130(10):868-877. doi: 10.20452/pamw.15371. # indicates that these authors contributed equally to this work. Received: April 8, 2020. Accepted: April 29, 2020.
- Publication 3: Carlos Bravo-Pérez, María Eugenia de la Morena-Barrio, Belén de la Morena-Barrio, Antonia Miñano, José Padilla, Rosa Cifuentes, Pedro Garrido, Vicente Vicente, Javier Corral. Molecular and clinical characterization of transient antithrombin deficiency: A new concept in congenital thrombophilia. Am J Hematol. 2022. 97(2):216-225. doi: 10.1002/ajh.26413. Received: November 9, 2021. Accepted: November 16, 2021.
- Publication 4: María E de la Morena-Barrio #, Réka Gindele #, Carlos Bravo-Pérez #, Péter Ilonczai #, Isabel Zuazu, Marianna Speker, Zsolt Oláh, Juan J Rodríguez-Sevilla, Laura Entrena, Maria S Infante, Belén de la Morena-Barrio, José M García, Ágota Schlammadinger, Rosa Cifuentes-Riquelme, Asunción Mora-Casado,

Antonia Miñano, Jose Padilla, Vicente Vicente, Javier Corral, Zsuzsanna Bereczky. High penetrance of inferior vena cava system atresia in severe thrombophilia caused by homozygous antithrombin Budapest 3 variant: Description of a new syndrome. Am J Hematol. 2021;96(11):1363-1373. doi: 10.1002/ajh.26304. # indicates that these authors contributed equally to this work. Received: April 26, 2021. Accepted: July 27, 2021.

- Publication 5: Carlos Bravo-Pérez 1, Maria E de la Morena-Barrio 1, A Palomo 2, L Entrena 3, B de la Morena-Barrio 1, J Padilla 1, A Miñano 1, E Navarro 1, R Cifuentes 1, J Corral 1, V Vicente 1 Genotype-phenotype gradient of SERPINC1 variants in a single family reveals a severe compound antithrombin deficiency in a dead embryo. Br J Haematol. 2020;191(1):e32-e35. doi: 10.1111/bjh.16963. Received: May 24, 2020. Accepted: June 22, 2020.
- Publication 6: A pilot study on the impact of congenital thrombophilia in COVID-19. Maria Eugenia de la Morena-Barrio #, Carlos Bravo-Pérez #, Belen de la Morena-Barrio, Christelle Orlando, Rosa Cifuentes, Jose Padilla, Antonia Miñano, Sonia Herrero, Shally Marcellini, Nuria Revilla, Enrique Bernal, Jose Miguel Gómez-Verdú, Kristin Jochmans, María Teresa Herranz, Vicente Vicente, Javier Corral, María Luisa Lozano. Eur J Clin Invest.. 2021;51(5):e13546. doi: 10.1111/eci.13546. # indicates that these authors contributed equally to this work. Received: Feb 18, 2021. Accepted: March 16, 2021.

Publications 1 and 2 consist of two narrative reviews on antithrombin deficiency, performed by the doctorate student, that were the germ of the general introduction of this dissertation, and also helped to establish its aims. Here, we showed a practical review of antithrombin deficiency, focused on molecular basis, diagnostic procedures, prognostic implications, and clinical management of this severe thrombophilia. Many of

the puzzling questions tackled in this thesis are highlighted in this works. As the first author of this reviews, the doctorate student performed extensive bibliographic search, integrated this information with his own investigations, created the tables and figures and wrote and revised the manuscript draft and its later amendments.

Publication 3 constitutes the first original research of this doctoral thesis, and addresses a diagnostic problem. In this work, we focused on the identification and characterization of cases with at least a positive finding by functional methods supporting antithrombin deficiency that however was not confirmed by a second analysis in other laboratory or in other sample from the same patient: an atypical laboratory scenario that we designated transient antithrombin deficiency. As the first author of this work, the doctorate student performed extensive bibliographic search, assisted local patients, performed experiments, analyzed data, made the figures, and wrote and revised the manuscript draft and its later amendments.

Publications 4 and 5 provide novel and strong clinical evidence supporting the impact of severe antithrombin deficiency in early human development. Publication 4 is a case-based multicenter study, in which we demonstrated the association of homozygous antithrombin Budapest 3 variant (p.Leu131Phe) with inferior vena cava atresia, a congenital malformation that may arise as the result of intrauterine/ perinatal thrombosis facilitated by antithrombin deficiency. Publication 6 is the study of an exceptional thrombophilic family with antithrombin deficiency and history of recurrent miscarriage, that revealed an extremely severe antithrombin deficiency in a dead embryo, caused by the compound heterozygous state for antithrombin Budapest 3 and a variant causing quantitative deficiency. As a co-first author of publication 4 and first author of publication 5, the doctorate student performed extensive bibliographic search, assisted

local patients, performed experiments, analyzed data, made the tables and figures, and wrote and revised both manuscript drafts and their later amendments.

Finally, publication 6 consists of the last original research of this dissertation, published as a brief report, and is motivated by the SARS-CoV-2 pandemic. In this pivotal study, which was the result of a multicenter task force, we first addressed the role of congenital thrombophilia in COVID-19. This work will encourage further research to clarify the biological and clinical consequences of inherited thrombophilic states in SARS-CoV-2 infection, as well as to guide the optimal approach to manage anticoagulation in these cases. As a co-first author, the doctorate student performed extensive bibliographic search, performed experiments, analyzed data, made the tables and figures, and wrote and revised both manuscript drafts and their later amendments.

The regulation of this modality of thesis is detailed in the Doctorate Studies Policy of Universidad de Murcia (Reglamento por el que se Regulan las Enseñanzas Oficiales de Doctorado de la Universidad de Murcia).[11]

Chapter 1
General introduction

1. General introduction

1.1. Antithrombin molecule

Antithrombin is a plasma glycoprotein of hepatic origin. The mature protein has 432 amino acids (aa), a molecular weight of 58 KDa, a plasma concentration of 150 μg/mL and a half-life of 65 hours.[6,12] The gene encoding antithrombin, SERPINC1, is situated on the long arm of chromosome 1 (1q23-q25.1), it is 13.5 Kb length and has seven exons and six introns (Figure 3A).[9,13] The gene encodes a precursor of 464 residues, that derives into the mature protein after removal of a N-terminal signal peptide with 32 aa. Antithrombin also contains six cysteine residues, which establish three disulphide bonds (Cys40-160, Cys53-127, and Cys279-462), and four asparagine residues (Asn128, 167, 187 and 224) that are targets for N-glycosylation (Figure 3B).[12] Since Asn167 is inefficiently glycosylated, antithrombin has two different physiologic glycoforms in plasma, α- and β-antithrombin, with four and three glycans, respectively.[14]

Antithrombin may also be designated serpin C1, since it is the first member of the clade C of serpin superfamily (serpin: serine protease inhibitor).[15] The protein is spontaneously folded into a highly conserved tertiary structure consisting of three β-sheets (A-C), nine α-helices (A-I) and a reactive centre loop (RCL).[12] Serpin structure enables protease inhibition by an original mousetrap-like mechanism.[16] Like all inhibitory serpins, antithrombin requires two sine qua non conditions for coagulation protease inhibition. Firstly, an exposed reactive site, that carries the dipeptide P1-P1' (p.Arg425-Ser426 in antithrombin) that mimics the target residues of the serine proteases.[16,17] Secondly, a native metastable conformation, what means that, unlike most proteins,[18] serpins spontaneously fold into an energetically suboptimal or stressed (S) state, in which β-sheet A consists of five strands and the reactive site forms an exposed loop that is C-terminal-anchored by strands s1C and s4B-5B.[19]

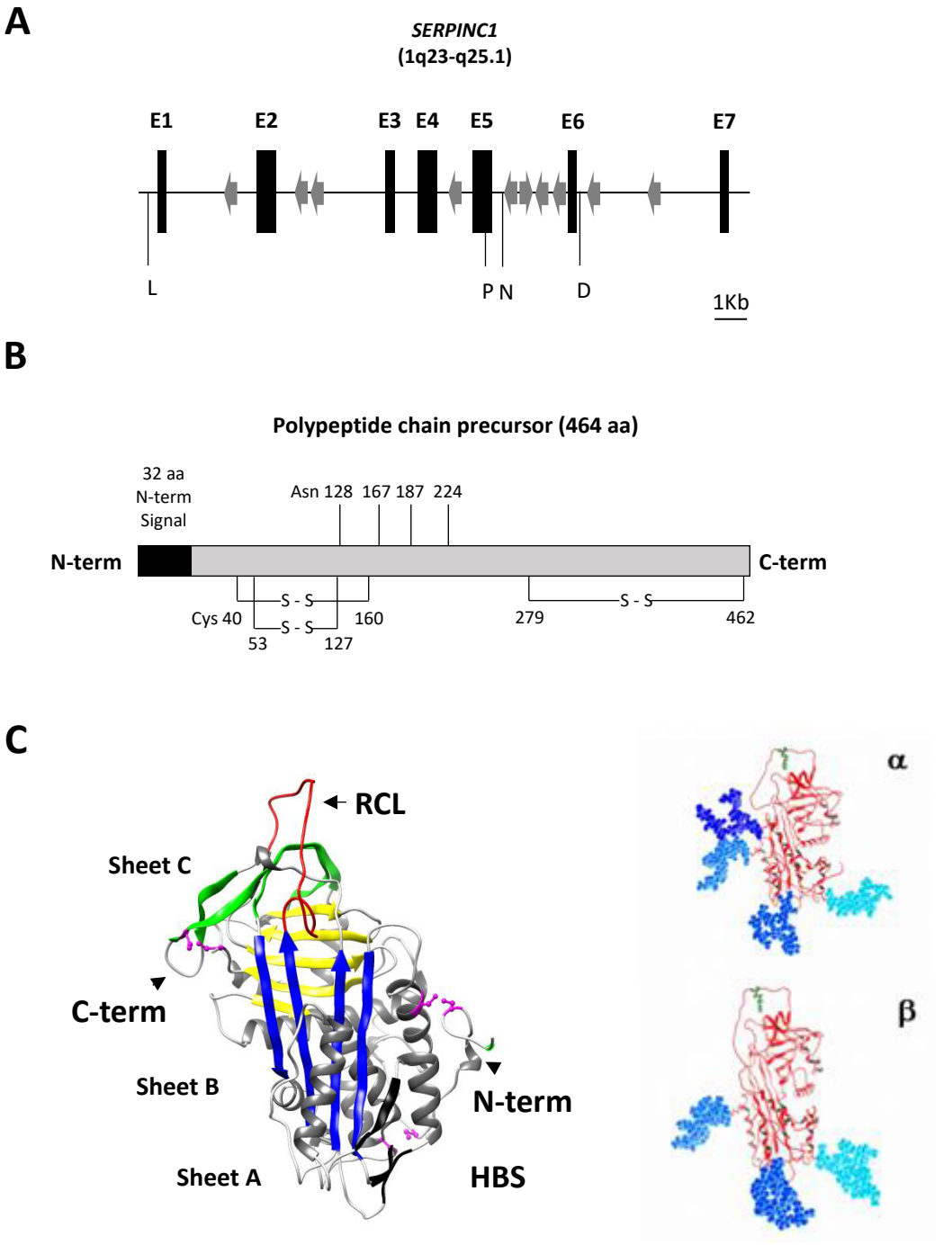

Figure 3. Antithrombin gene, polypeptide chain and structure. A) Antithrombin gene (SERPINC1). Seven exons **(**E1-E7), Alu sequences (grey arrows) and polymorphic regions (L, P, N and D) are represented. Modified from Biochemistry 1993;32:4216-4224.[9] **B) Antithrombin polypeptide precursor (464 aa).** N-term signal peptide (32 aa), asparagine residues for N-glycosylation (Asn128, 167, 187 y 224) and cysteine residues and disulphide bonds (Cys40-160, Cys53-127, Cys279-462) are represented. **C) Antithrombin structure and N-glycoforms.** Main **s**econdary structures and functional regions of antithrombin (PDB: 2ANT) are coloured: β-sheets A, B and C (blue, yellow and green, respectively)**,** α-helix A-I (dark grey) and the RCL (red). Cysteine residues are also shown in the main structure (magenta balls and sticks). The two N-glycoforms of antithrombin, α and β, with four and three glycans, respectively (blue balls and sticks), are represented at the side. aa: amino acids. AT: antithrombin. C-term: C-terminal. N-term: N-terminal. HBS: Heparin binding site. PDB: Protein Data Bank (https://www.wwpdb.org/). RCL: reactive centre loop.

More stable or relaxed (R) conformations, such as cleaved or latent states, imply the sequestration of the RCL into the centre of β-sheet A, what results in a six-stranded-β-sheet A expansion (Figure 4).[19–21]

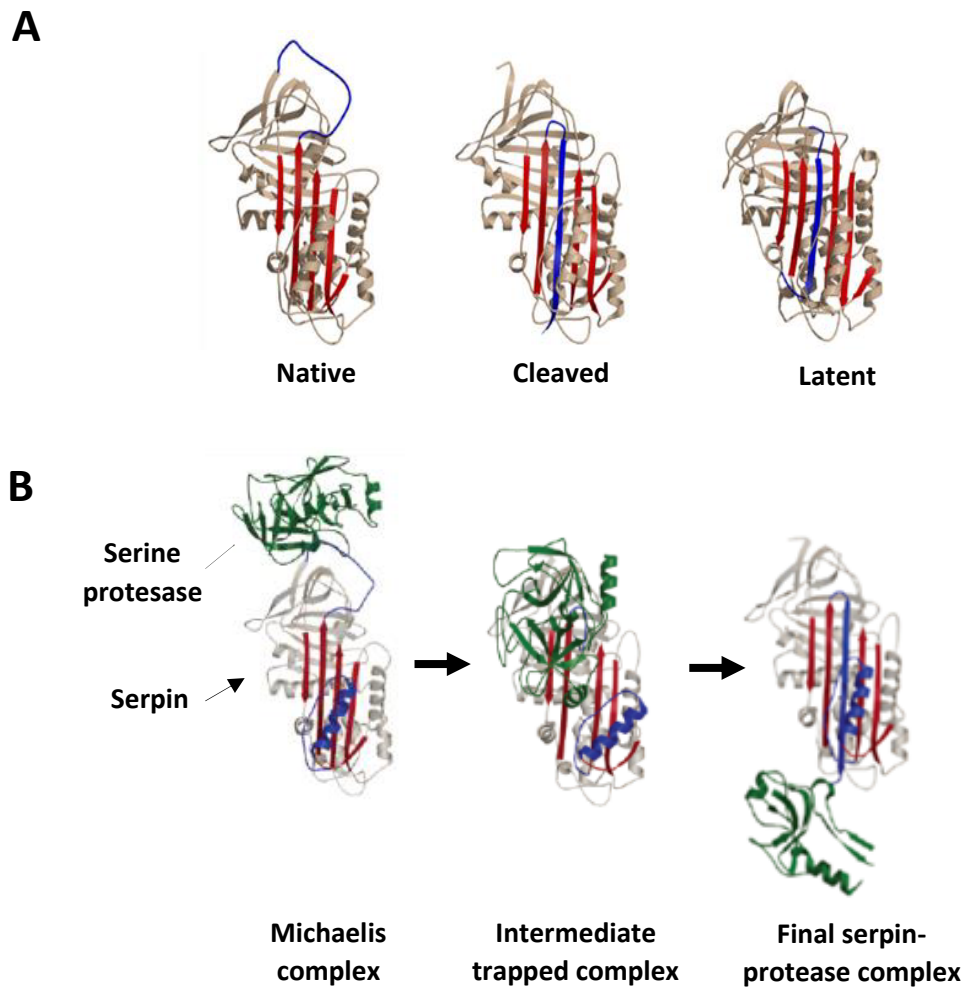

Figure 4. Serpin conformation and mousetrap-like inhibitory mechanism. A) Main serpin conformations. Native (α1-antitrypsin, PDB: 1QLP), cleaved (α1-antitrypsin, PDB: 7API) and latent (PAI-1, PDB: 1CG5). Note that the β-sheet A (red) differs in the presence of 5 strands (native) or 6 strands (cleaved, latent) by incorporation of the RCL (blue). Taken from Prog Mol Biol Transl Sci. 2011;99:185–240.[20] **B) Mousetrap-like inhibitory mechanism (trypsin inhibition by α1-antitrypsin).** Main initial, intermediate and final serpin – protease complexes are shown. β-sheet A (red), RCL (blue) and protease (green) are highlighted. Modified from Gettins PGW et al. Chem. Rev. 2002;102:4751-4804.[21]

PAI-1: plasminogen activating inhibitor-1. PDB: Protein Data Bank (https://www.wwpdb.org/). RCL: reactive centre loop.

Under these principles, by the same time antithrombin is cleaved and forms a covalently linked, transient complex with the target coagulation protease, stressed to relaxed transition occurs, and the release of energy results in protease deformation and inhibition with a 1:1 stoichiometry. The final complexes are thermodynamically stable (for days), so that they are cleared from plasma before their dissociation.[16,17,19]

Unlike most serpins, the RCL of native antithrombin is only partially exposed, and it becomes totally accessible after heparin binding, what increases up to 1000-fold its inhibitory activity.[22] The heparin binding site (HBS) of antithrombin is formed by positively charged residues mainly located on the N-terminal end and α-helix D.[23]

1.2. Antithrombin deficiency

Antithrombin deficiency, defined by low plasma antithrombin activity (normal range: 80 - 120%), is associated with an increased risk of thrombosis.[4,24,25] Antithrombin deficiency can be a congenital disorder, due to gene variants, mainly but not exclusively in SERPINC1,[9,26] or acquired in distinct physiopathological scenarios:[27] cases with hepatic dysfunction (cirrhosis or L-asparaginase therapy),[28,29] massive thrombin generation (acute thrombosis or disseminated intravascular coagulation)[30] or protein loss (renal disease with nephrotic syndrome or use of extracorporeal circulation devices).[31,32]

Congenital antithrombin deficiency was the first major thrombophilia reported, and the most severe so far.[5] It is an autosomal dominant disorder with incomplete penetrance and variable expressivity.[4] Classical studies reported a very low prevalence of antithrombin deficiency in the general population (0.02 - 0.2%).[4] Nonetheless, many works suggest that it might be underestimated, due to the limitation of current diagnostic methods and its molecular and clinical heterogeneity.[33–38]

1.3. Genetics of antithrombin deficiency

To date, more than 400 pathogenic mutations in SERPINC1 have been reported (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SERPINC1).[39] Antithrombin deficiency can be classified into two groups: type I (quantitative deficiency) and type II (qualitative deficiency). Additionally, type II defects can be divided into reactive site (II/RS), heparin binding site (II/HBS) and pleiotropic effect (II/PE) subtypes.[40]

Type I deficiency is usually caused by severe SERPINC1 gene variants in heterozygous state, such as nonsense or splicing variants, insertions/ deletions (INDEL) and structural variants.[27] On the contrary, type II deficiency is commonly due to missense variants with a minor effect on antithrombin structure and function. Nevertheless, some missense mutations are also associated to type I deficiency, by means of RNA instability or abnormal splicing, or protein polymerization.[40,41]

Gene variants in other loci rather than SERPINC1, involved in protein transcription, folding or post-translational modifications, might theoretically lead to a defective status of antithrombin with no mutations in SERPINC1.[9] Indeed, recent works of our group support that a relevant proportion of cases with antithrombin deficiency with no variants in SERPINC1 can be caused by N-glycosylation defects.[42]

1.4. Thrombosis associated to antithrombin deficiency

Antithrombin deficiency increases the risk of thrombosis.[5] Venous thromboembolism (VTE) is primarily related to antithrombin deficiency.[10] The odds ratios (OR) for VTE estimated by the first case series were extremely high (OR: 50, 95% CI: 14.17-171.16).[43] Further works have reported more moderate but still high risks (OR: 14, 95% CI: 5.5-29.0),[25] probably due to the inclusion of patients with a more heterogeneous genotype. Anyway, antithrombin deficiency remains as the most severe thrombophilia,[5,25,44–46] with an estimated annual risk for VTE of up to 2.3% (95% CI:

0.2-6.5%).[25] Deep venous thrombosis (DVT) in the lower extremities and/ or pulmonary embolism (PE) are the most common clinical manifestations, but thrombosis in atypical localizations, such as the vena cava or splanchnic or cerebral veins, may also occurred.[47–49]

Thrombosis is a complex disease, and antithrombin deficiency shows a remarkable heterogeneity within a continuum, from asymptomatic subjects who are incidentally diagnosed, to the most severe cases with early and recurrent thrombosis in all carriers of a gene variant within a family. Both environmental and other genetic risk factors may modulate the thrombotic phenotype associated to antithrombin deficiency.[33,44] On the one hand, external risk factors can be identified in approximately 50% of patients with VTE and severe thrombophilia. Common risk factors are trauma, immobilization, surgery, hormone therapy, pregnancy and postpartum.[44,50] Additionally, the type of gene variant, and so the type of deficiency, significantly influences the risk of thrombosis. Missense variants associated to type II deficiency normally confer a lower risk than nonsense mutations or INDELs, linked to type I deficiency. Complete absence of antithrombin, caused by quantitative-deficiency variants in homozygosis, are lethal.[51] However, missense variants causing type II deficiency in homozygous state might be compatible with life, but lead to a very severe clinical phenotype, with paediatric or even perinatal thrombosis.[41]

1.5. Diagnosis of antithrombin deficiency

Antithrombin deficiency diagnosis is based on the classical functional-biochemical-genetic sequential algorithm (Figure 5).[9]

1.5.1. Functional and antigenic methods

The first diagnostic step consists of evidencing low antithrombin activity in plasma (<80% of the normal range) by performing functional assays based on amidolytic

methods. Since these functional alterations are constitutive in antithrombin deficiency, more than one defective measure at different times are necessary to verify a definitive diagnosis.[27,52] Current functional methods are based on chromogenic substrates that evaluate anti-FIIa or anti-FXa anticoagulant activity of antithrombin.[22] Normally, anti-FXa assays are preferred rather than anti-FIIa, as they avoid the (minor) interference of heparin cofactor II (HCII), a second anticoagulant serpin that also inhibits thrombin.[38,53] However, it should be highlighted that current functional methods fail to detect some type II/RS and HBS mutations in SERPINC1 (Table 1).[27,53–62]

Table 1. SERPINC1 variants hardly detected by functional methods

Variant (Name)	Type of deficiency	Functional consequences	Technical limitations
p.Pro73Leu (Basel)	Type II/HBS	Defective heparin binding	Anti-FIIa Anti-FXa (long incubation)
p.Asn77His	Type II/HBS	Defective heparin binding	Anti-FIIa Some anti-FXa
p.Arg79Cys (Toyama)	Type II/HBS	Defective heparin binding	More difficult by anti-FIIa
p.Arg79His (Rouen I)	Type II/HBS	Defective heparin binding	All methods
p.Leu131Phe (Budapest 3)	Type II/HBS	Defective heparin binding	Anti-FIIa
p.Lys146Glu (Dreux)	Type II/HBS	Defective heparin binding	Long incubations
p.Gln150Pro (Vienna)	Type II/HBS	Defective heparin binding	Long incubations
p.Arg161Gln (Geneva)	Type II/HBS	Defective heparin binding	Long incubations
p.Ala416Ser (Cambridge II)	Type II/RS	Thrombin substrate	Anti-FXa
p.Gly424Asp (Stockholm)	Type II/RS	Impaired interaction with protease	Anti-FXa
p.Arg425His (Glasgow)	Type II/RS	Impaired interaction with protease	Anti-FIIa (long incubation)
p.Ser426Leu (Denver)	Type II/RS	Impaired interaction with protease	Anti-FXa

Modified from: Corral J et al. Thrombosis Research. 2018; 169: 23-29.[9]

FIIa: thrombin. FXa: activated factor X. HBS: heparin binding site. RS: reactive site.

Antigenic assays are useful to distinguish between type I or type II deficiency.[52] Different methods may measure antigen levels of antithrombin in plasma, such as enzyme-linked immunoassay (ELISA), rocket immunoelectrophoresis, radial immunodiffusion or immunoturbidimetric methods. Western blot analysis using different electrophoretic conditions may better characterize the type of deficiency.[9] Furthermore, in cases with type II deficiency, progressive activity assays and analysis of heparin affinity by crossed immunoelectrophoresis (CIE) may enable a complete diagnosis of the functional defect.[9]

1.5.2. Molecular analysis

An integral diagnosis of antithrombin deficiency should include genetic studies. As clinical heterogeneity among cases with antithrombin deficiency is remarkable, even with similar or identical functional activity, the genetic analysis would assist to a correct diagnosis and to better define the prognosis.[63]

According to a current algorithm for genetic analysis of cases with antithrombin deficiency proposed by our group (Figure 5),[9,64,65] the first step is performing Sanger sequencing of the seven exons and flanking regions of SERPINC1, as this approach would identify molecular alterations in the majority of subjects (up to 70 or 80%).[9,66] Next generation sequencing (NGS) might be alternatively used.[9] For cases with negative results, multiplex ligation-dependent probe amplification (MLPA) could be used to detect structural variants, that would constitute 2 to 5% of cases [66,67]. Whole gene sequencing, including SERPINC1 promoter and introns, might uncover mutations in regulatory regions, which also may represent a small proportion of cases.[68,69]

Finally, cases with persistent negative findings in SERPINC1 analysis might be tested for N-glycosylation defects, as this mechanism could underline up to 27% of these cases.[42] Analysis of N-glycoforms of antithrombin, and of other plasma glycoproteins,

by electrophoretic methods or by high-performance liquid chromatography (HPLC), is only available in centres of reference, but it might be of research interest, and also clinical one through prognostic or therapeutic implications.[9]

Overall, following this algorithm, in the largest series of patients, including ours, a molecular basis is identified in approximately 85% of cases.[9,65]

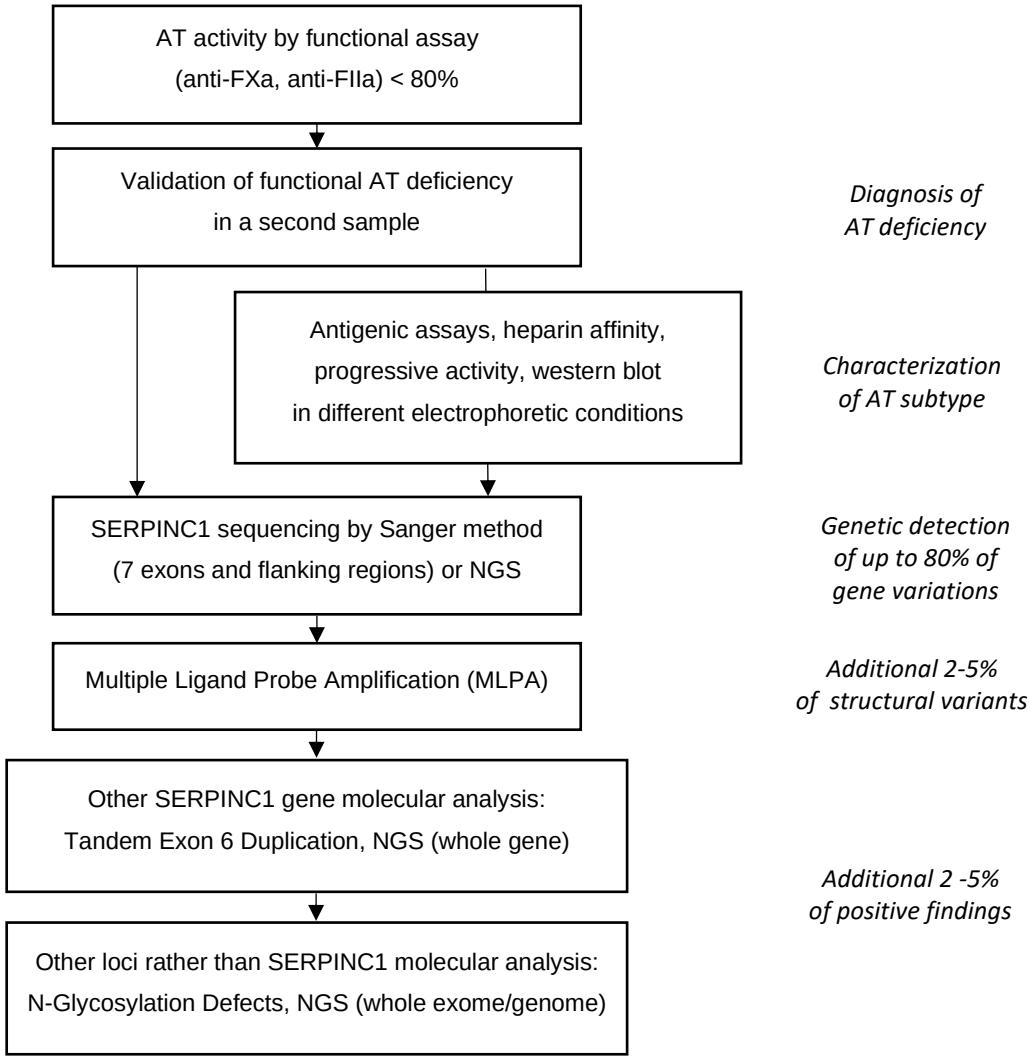

Figure 5. Algorithm for diagnosis and molecular characterization of antithrombin deficiency. Taken from Bravo-Perez C et al. Expert Rev Hematol. 2019. 12(6):397-405.[64,65]

AT: Antithrombin. FIIa: thrombin. FXa: activated factor X. NGS: Next Generation Sequencing.

1.6. Clinical management of patients with antithrombin deficiency

Anticoagulant therapy in patients with antithrombin deficiency essentially follows the standard recommendations for other inherited major thrombophilias,[70,71] but with the particularity that antithrombin concentrates might be used for both VTE treatment and prevention (Table 2).[72–75]

During an acute episode of VTE, antithrombin concentrates may be administered, combined to heparin, specifically in cases of severe/ recurrent thrombosis or heparin resistance.[72,73,76]

After the acute phase, antithrombin supplementation may be stopped and switching to oral therapy is performed. Antivitamin K (AVK) anticoagulants are usually used for this purpose, since they accumulate more clinical experience, but the evidence about the use of direct oral anticoagulants (DOACs) is constantly growing.[77–79]

Currently, there are two different antithrombin concentrates available for clinical use: the plasma derivative ThombateIII®[80] and the recombinant product ATryn®.[81] However, these agents are not equivalent and have not been compared, so they cannot be indistinctly used. While both products are indicated for thromboprophylaxis, only the plasmatic concentrate is indicated for the treatment of VTE (Table 2).[64,80,81]

Long-term anticoagulation aims prevention of thrombosis recurrence,[70,71] and it might be individualized depending on clinical and molecular data if available.[64,65]

Subjects with no personal history of thromboembolism (or those with a positive history of thrombosis but not ongoing long-term anticoagulation), might beneficiate from thromboprophylaxis with heparin and/ or antithrombin supplementation at high-risk scenarios,[44,49,50,74,76,82] particularly surgery[83–85] and pregnancy or postpartum.[86–89] Management of cases at paediatric age might be individualized.[90–92]

Table 2. Comparison of FDA-approved, human antithrombin concentrates

	Trombate III®	**ATryn®**
FDA approval	1991	2009
Production	Human pooled plasma derivative	Recombinant DNA technology
Indications	In congenital AT deficiency for: -Prevention of venous thrombosis. -Treatment of venous thrombosis.	In congenital AT deficiency for prevention of venous thrombosis
Half-life	2.5 - 3.8 days	12 - 18 h
Administration	IV infusion in 10 – 20 min, every 24 h	IV infusion in 15 min, followed by continuous IV perfusion
Loading dose	$\frac{(\text{Target AT\%} - \text{Baseline AT\%}) \times \text{Body Weight}}{1.4\%}$ †	$\frac{(100 - \text{Baseline AT\%}) \times \text{Body Weight}}{\begin{matrix}2.3\% \text{ for surgery} \\ 1.3\% \text{ for pregnancy}\end{matrix}}$ †
Maintenance dose	Initially, loading dose x 0.6. Subsequently, adjust with "loading dose" formula and according to actual AT %	Continuous perfusion dose (UI/h)= $= \frac{(100 - \text{Baseline AT\%}) \times \text{Body Weight}}{\begin{matrix}10.2\% \text{ for surgery} \\ 5.4\% \text{ for pregnancy}\end{matrix}}$ Subsequently, adjust by adding/ subtracting maint. dose x 0.3
Dose adjustment and monitoring	Monitor AT activity pre-injection, 20 min later and at least every 12 h post-injection	Initially, monitor AT activity every 2 h, when the target range is achieved every 6 - 12 h
Target AT activity	80 – 120%	
Adverse reactions	Hypersensitivity reactions, transmission of infectious agents	Hypersensitivity reactions

†Body weight in Kg. The dose calculation is based on an expected incremental in vivo recovery (%/ unit/ Kg) above baseline or actual levels (1.4% for Trombate III®, different for ATryn®, according to loading/ maintenance and surgery/ pregnancy context).

Taken from Bravo-Perez C et al. Expert Rev Hematol. 2019. 12(6):397-405.[64,65]

AT: Antithrombin. FDA: Food and Drug Administration. IU: International Units. h: hours. min: minutes.

1.7. Atypical scenarios for antithrombin deficiency

Atypical manifestations beyond VTE have been reported in cases with antithrombin deficiency. Here, we focus on arterial thrombosis, obstetric adverse outcomes, and multi-system syndromes.

1.7.1. Arterial thrombosis

Atherothrombosis (ischemic stroke, coronary artery disease, etc) is not characteristic in severe thrombophilia, but it has been suggested that the hypercoagulable state in subjects with deficiency of antithrombin, and other natural anticoagulants, may also contribute to the development of arterial events.[93,94] Several attempts have not succeeded to demonstrate that patients with antithrombin deficiency have a significant risk for arterial thrombosis,[95–97] but this might have been explained by the apparent low frequency and high molecular heterogenicity of SERPINC1 defects. Anyway, it has been reported that patients with antithrombin deficiency also have arterial thrombosis.[33,98] Notably, this observation is more patent in carriers of mild, type II variants, suggesting that long-term anticoagulant therapy, initiated after a VTE episode in the more severe cases, secondary prevents later arterial events. Indeed, heterozygous subjects for type II/HBS variants, associated to the lowest risk of VTE, have the highest arterial/ venous thrombosis ratio.[99] Illustratively, this is the case of antithrombin Budapest 3 (p.Leu131Phe). Whereas homozygous carriers of this type II/HBS variant have early/ recurrent VTE, heterozygous ones have an incidence of arterial thrombotic events as high as 10%.[99]

1.7.2. Obstetrical adverse outcomes

Some case series report that antithrombin deficiency is associated to a high risk of obstetrical complications in women, mainly embryo-foetal losses, but also pre-

eclampsia, intrauterine growth restriction, abruptio placentae and foetal distress.[86,87,100,101] Despite antithrombin is increasingly related to development and angiogenesis,[102] a thrombotic component seems to be essential for adverse obstetrical outcomes in this context, since these complications are more frequent in the more severe cases, and women that receive thromboprophylaxis seem to have less obstetric events.[89,101]

1.7.3. Multi-system syndromes

Molecular study of patients with antithrombin deficiency of apparently unknown genetic basis (no variants in SERPINC1) has enabled the identification of new molecular defects and pathogenic mechanisms.[9] Remarkably, in these cases, thrombosis is one of a myriad of clinical manifestations that may hide a clinically relevant deficiency of antithrombin.

Chromosome 1q24q25 microdeletion syndrome. Several works have reported cases with interstitial deletions of 1q24q25, who have antithrombin deficiency due to a total or partial deletion of SERPINC1, located in this chromosomic region, but also syndromic phenotype, depending on the extension of the structural variant.[103] Commonly, these cases have early developmental delay, psychomotor retardation and dysmorphic features, but more complex manifestations have also been described, including autoimmune disease,[104] hypopituarism and infiltrative lipomatosis.[105]

Congenital disorders of glycosylation. Discovered in the 1980s by Jaeken et al.,[106] congenital disorders of glycosylation (CDG) is a large and diverse category of genetic, metabolic disorders that are due to defects in the synthesis or addition of glycans onto proteins and lipids.[106] As a main post-translational modification, the lack of N- or O-glycosylation in CDGs causes severe syndromes, with growth and psychomotor retardation, dysmorphic phenotype and multi-organ failure.[106] Moreover, the

impairment of N-glycosylation leads to multiple and complex haemostatic abnormalities, among which thrombosis and antithrombin deficiency, due to antithrombin hypoglycosylation, has consistently been described.[107]

Classically, CDGs are identified during infancy or childhood, clued by severe developmental or multi-system manifestations that predominate over thrombotic and haemostatic ones. However, recent works show that CDGs seem to exert higher heterogeneity than expected, modified by genetic and environmental factors.[42,108] Therefore, mild cases, not so suggestive of a N-glycosylation defect, might have remained undiagnosed until a thrombotic event during adulthood occurs. In fact, recent works leaded by our group demonstrate that a relevant proportion of cases with antithrombin deficiency and no variants in SERPINC1 is constituted by pauci-symptomatic forms of CDGs, dominated by antithrombin deficiency as the result of hypoglycosylation of this molecule.[42,108] Additionally, the diagnosis of these forms of CDG might be applied to the clinic, since it could lead to therapeutic benefits.[109] The most illustrative example is the CDG caused by mannose-6-phosphate isomerase gene (MPI) variants, a rare but probably underestimated disorder, that is associated to inflammatory-bowel-disease-like symptoms and antithrombin deficiency. Oral D-mannose supplementation, which bypasses the enzymatic stop, may simply restore all clinical and laboratory manifestations in these cases, including the normalization of antithrombin levels and the consequent antithrombotic protection.[110]

Chapter 2
Aims and lines of research

2. Aims and lines of research

2.1. General aim

The final aim of this research was to uncover new mechanisms and roles of antithrombin deficiency, in order to improve the diagnosis and management of this disorder. We addressed this objective with an original approach, the characterization of cases with antithrombin deficiency and atypical manifestations or emergent health problems, such as SARS-CoV-2 infection.

2.2. Lines of research

This thesis had four specific aims or lines of research:

2.2.1. Line of research 1

To identify and characterize cases with transient antithrombin deficiency. In accordance with the current diagnostic algorithm of antithrombin deficiency, previously detailed,[60] genetic analysis of SERPINC1 is restricted to cases with at least two defective antithrombin values by using functional assays, or with other relatives carrying this disorder. This approach has enabled the identification of the molecular basis in up to 80% of cases with antithrombin deficiency, and rendered plenty of both biochemical and genetic knowledge about antithrombin.[9,66] Furthermore, N-glycosylation defects underlie a proportion of cases with antithrombin deficiency that is not explained by SERPINC1 variants.[42]

However, antithrombin deficiency diagnosis still encloses puzzling questions. On the one hand, antithrombin is a crucial anticoagulant, and mild disturbances on this serpin will significantly increase the risk of thrombosis.[111] On the other hand, the prevalence of antithrombin deficiency is excessively low, not only in general population, but also among consecutive patients with venous thromboembolism.[112] Indeed, the use of NGS

in consecutive patients with VTE suggests that antithrombin deficiency might be and underestimated disorder, that silently increases the risk of thrombosis.[33–38] False negative results by using functional assays for antithrombin deficiency screening have been reported, all involving type II/RS and HBS variants.[58]

With this background, we hypothesised the existence of an atypical form of antithrombin deficiency that would be elusive to an easy diagnosis to the first functional step of the classic diagnostic strategy: what we have called transient antithrombin deficiency. In line of research 1, our aim was to identify cases with transient antithrombin deficiency and to characterize the underlying molecular mechanisms involved.

2.2.2. Line of research 2

To assess the frequency of inferior vena cava system defects in homozygous carriers of antithrombin Budapest 3 variant (p.Leu131Phe). The development of the venous system in the human embryo takes place during the 6^{th}-8^{th} gestational weeks. The inferior vena cava (IVC) results from the fusion and regression of three paired embryonic veins: the posterior cardinal, subcardinal and supracardinal veins. The alteration of this complex process may cause multiple congenital IVC malformations.[113]

Atresia of the infrarenal segment of the IVC is an extremely rare anomaly in the general population (prevalence of 0.07%).[114] Except for renal hypoplasia or aplasia, no other associated defects have been reported in these cases. Thus, it is usually diagnosed by incidental finding on clinical radiology exams.[114] As a single embryonic event does not fully explain this malformation, some authors suggest that it may be an acquired defect.[115] In 1980, Milner and Marchan were the first authors suggesting that IVC atresia was not caused as the result of a primary developmental failure of an embryonic venous system, but as the consequence of a thrombotic event on the immature vessels

during the intrauterine or perinatal period,[116] hypothesis also supported thereafter by other works.[113,117–120]

To our knowledge, only one study questions whether IVC atresia might be hereditary, based on two cases who had family aggregation of this vascular anomaly from a cohort of 163 subjects with IVC atresia.[121] Unfortunately, no genetic defect was reported in any of these two cases.[121]

Within our cohort, the identification of IVC atresia in a case with early idiopathic VTE and severe antithrombin deficiency caused by the homozygous antithrombin Budapest 3 variant (p.Leu131Phe), encouraged us to evaluate the role of this severe thrombophilia in this rare vascular malformation. Line of research 2 aimed to assess the frequency of IVC system defects in homozygous carriers of antithrombin Budapest 3 variant.

2.2.3. Line of research 3

To characterize a thrombophilic family with antithrombin deficiency and a strong history of embryo-foetal and neonatal deaths. Antithrombin deficiency is associated to a high risk of adverse obstetrical outcomes.[86,87,100,101] Antithrombin is increasingly related to development and angiogenesis.[102] Complete absence of antithrombin is lethal in animal models,[51] and this fact suggests that the thrombin/ antithrombin axis might play a relevant role in embryonic states. However, the real impact of antithrombin deficiency in human development has only been pointed out by research conducted in animal models or indirect clinical observations.[86,87,100,101] Thus, in this context, the analysis of natural mutants of antithrombin within particularly severe cases constitutes an ideal platform to further investigate the potential association of antithrombin deficiency and embryonic lethality in humans.

Within our cohort, we identified a unique thrombophilic family with different SERPINC1 defects causing antithrombin deficiency, that had also a strong history of

embryo-foetal and neonatal deaths. In line of research 3, we aimed to characterize the genotype-phenotype correlation of SERPINC1 variants within the thrombotic and obstetric complications observed in this family.

2.2.4. Line of research 4

To analyse the association between inherited thrombophilias and manifestations and severity of SARS-CoV-2 infection. Complex interactions underlie the hypercoagulable state and strong tendency for thrombosis reported in SARS-CoV-2 infection, but the individual factors that predispose to these adverse outcomes have to date not been clearly determined.[122] Inherited thrombophilic defects significantly increase the risk of thrombosis. Both mild prothrombotic polymorphisms, such as FV Leiden and prothrombin G20210A, and major thrombophilia caused by the deficiency of the natural anticoagulants antithrombin, protein C and protein S, are associated to early and recurrent thrombosis, particularly when combined with additional factors.[123] Consequently, and as suggested in some editorials and reviews, congenital thrombophilia might exacerbate the already increased risk for thrombosis in COVID-19.[124] However, no single report on SARS-CoV-2 infection or COVID-19 contains data regarding inherited thrombophilia, apart from the description of a palmar digital vein thrombosis in a subject who was heterozygous for FV Leiden.[125] Thereby, during the first pandemic peak of SARS-CoV-2 in Spain, line of research 4 was incorporated to this thesis to investigate the potential association between inherited thrombophilias, particularly antithrombin deficiency, and COVID-19 complications.

Chapter 3
Summary of publications

3. Summary of publications

3.1. Publication 1: Management of antithrombin deficiency: an update for clinicians

3.1.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Vicente Vicente, Javier Corral.
- **Journal:** Expert Review of Hematology.
- **Year:** 2019.
- **Volume (Issue):** 12 (6). **Pages:** 397-405.
- **DOI:** 10.1080/17474086.2019.1611424.
- **ISSN:** 1747-4086.
- **Impact factor (JCR, 2019):** 2.573.
- **Category:** Haematology. **Position:** 42/76. Quartile 3.
- **Received:** 19 Feb 2019. **Accepted:** 23 Apr 2019.

3.1.2. Brief description

In the present work, we globally reviewed the biology, genetics, diagnosis, and management of congenital antithrombin deficiency, and also discussed puzzling questions and future perspectives regarding this severe inherited thrombophilia. By conducting a deep bibliographic search and integrating it with data extracted from our own cohort, we conclude that although antithrombin deficiency exerts high clinical heterogeneity, many carriers will need careful and long-term anticoagulation and/ or thromboprophylaxis, especially in high-risk situations, such as surgery and pregnancy. Notably, antithrombin concentrates constitute a considerable arsenal for both treatment and prevention of VTE in subjects with antithrombin deficiency. Current evidence is based almost exclusively on retrospective case series, so an integrated functional,

biochemical and molecular characterization will be of clinical relevance and guide hematologists' individualized decisions.

3.2. Publication 2: Antithrombin deficiency as a still underdiagnosed thrombophilia: a primer for internists

3.2.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Maria E de la Morena-Barrio #, Vicente Vicente #, Javier Corral # (# indicates that these authors contributed equally to this work).
- **Journal:** Polish Archives of Internal Medicine.
- **Year:** 2020.
- **Volume (Issue):** 130 (10). **Pages:** 868-877**.**
- **DOI:** 10.20452/pamw.15371.
- **ISSN:** 0032-3772.
- **Impact factor (JCR, 2020):** 3.277.
- **Category:** Medicine, General & Internal. **Position:** 54/167. Quartile 2.
- **Received:** April 8, 2020. **Accepted:** April 29, 2020.

3.2.2. Brief description

In this review, we presented a practical summary of data regarding antithrombin deficiency to provide useful guidance for its proper diagnosis and management. We focused on its molecular basis, diagnostic procedures, prognostic implications, and clinical management. Unlike recent recommendations, we conclude that a complete diagnosis and molecular characterization of antithrombin deficiency is strongly recommended, in order to implement a proper, personalized management of a patient and their relatives, which should incorporate new sequencing methods not limited to the SERPINC1 gene testing. Interestingly, since the molecular landscape of antithrombin

deficiency is in continuous expansion, we specifically dedicate the last section of this paper to focus on atypical manifestations of antithrombin deficiency, beyond VTE: arterial thrombosis, obstetrical complications, and complex syndromes such as congenital disorders of N-glycosylation. Many of these atypical manifestations are studied in the lines of research of this thesis.

3.3. Publication 3: Molecular and clinical characterization of transient antithrombin deficiency: A new concept in congenital thrombophilia

3.3.1. Publication data

- **Authors:** Carlos Bravo-Pérez, María Eugenia de la Morena-Barrio, Belén de la Morena-Barrio, Antonia Miñano, José Padilla, Rosa Cifuentes, Pedro Garrido, Vicente Vicente, Javier Corral.
- **Journal:** American Journal of Hematology.
- **Year:** 2022.
- **Volume (Issue), Pages:** 97(2):216-225.
- **DOI:** 10.1002/ajh.26413.
- **ISSN:** 0361-8609.
- **Impact factor (JCR, 2020):** 10.047.
- **Category:** Haematology. **Position:** 7/76. Decile 1.
- **Received:** November 9, 2021. **Accepted:** November 16, 2021.

3.3.2. Brief description

Line of research 1 was addressed in this study. We analyzed a retrospective cohort of 444 consecutive, unrelated cases, with functional results supporting antithrombin deficiency in at least one sample (antithrombin activity < 80%), referred from 1998 to 2021 to our institution. Plasma antithrombin was evaluated by functional and

biochemical methods in at least two samples. SERPINC1 gene was analyzed by sequencing and MLPA. Hypoglycosylation was studied by electrophoresis and HPLC. In 260 of 305 cases (85.2%) with constitutive deficiency (activity <80% in all samples), a SERPINC1 (N = 250), or N-glycosylation defect (N = 10) was observed, while 45 remained undetermined. The other 139 cases had normal antithrombin activity (≥ 80%) in at least one sample, what we called transient deficiency. Sixty-one of these cases (43.9%) had molecular defects: 48 had SERPINC1 variants, with two recurrent mutations (antithrombin Cambridge II [p.Ala416Ser] N = 15, and antithrombin Dublin [p.Val30Glu] N = 12), and 13 had hypoglycosylation. Thrombotic complications occurred in transient deficiency, but were less frequent, latter-onset, and had a higher proportion of arterial events than in constitutive deficiency. Two mechanisms explained transient deficiency: the limitations of functional methods to detect some SERPINC1 variants and the influence of external factors on the pathogenic consequences of these mutations. Our study revealed a molecular defect in a significant proportion of cases with transient antithrombin deficiency, and changes the paradigm of thrombophilia, as the pathogenic effect of some mutations might depend on external factors and be present only at certain timepoints. Antithrombin deficiency is underestimated, and molecular screening might be appropriate in cases with fluctuating laboratory findings.

3.4. Publication 4: High penetrance of inferior vena cava system atresia in severe thrombophilia caused by homozygous antithrombin Budapest 3 variant: Description of a new syndrome

3.4.1. Publication data

- **Authors:** María E de la Morena-Barrio #, Réka Gindele #, Carlos Bravo-Pérez #, Péter Ilonczai #, Isabel Zuazu, Marianna Speker, Zsolt Oláh, Juan J Rodríguez-Sevilla, Laura Entrena , Maria S Infante, Belén de la Morena-Barrio, José M García,

Ágota Schlammadinger, Rosa Cifuentes-Riquelme, Asunción Mora-Casado, Antonia Miñano, Jose Padilla, Vicente Vicente, Javier Corral, Zsuzsanna Bereczky (# indicates that these authors contributed equally to this work).

- **Journal:** American Journal of Hematology.
- **Year:** 2021.
- **Volume (Issue):** 96(11). **Pages:** 1363-1373.
- **DOI:** 10.1002/ajh.26304.
- **ISSN:** 0361-8609.
- **Impact factor (JCR, 2020):** 10.047.
- **Category:** Haematology. **Position:** 7/76. Decile 1.
- **Received:** April 26, 2021. **Accepted:** July 27, 2021.

3.4.2. Brief description

Line of research 2 was addressed in this work. We evaluated the frequency of IVC atresia in patients homozygous for antithrombin Budapest 3 (p.Leu131Phe). We performed a cross-sectional study in a previously identified cohort of 61 subjects homozygous for the Budapest 3 variant, selected from 1,118 patients with antithrombin deficiency from two different populations: Spain (N = 692) and Hungary (N = 426). Image analysis included computed tomography (CT) and phlebography. Atresia of the inferior vena cava system was observed in 17 of 24 cases (70.8%) homozygous for antithrombin Budapest 3 with available image exam: 16 had absence of infrarenal IVC and one had atresia of the left common iliac vein. All cases with vascular defects had compensatory mechanisms, and seven also had other congenital anomalies. Short tandem repeat analysis (STR) supported the specific association of the vascular malformation with SERPINC1. We showed the first evidence of the association of a severe thrombophilia with IVC system atresia, supporting the possibility that a

thrombosis in the developing vessels was the reason for this anomaly. Our hypothesis-generating results encourage further studies to investigate severe thrombophilic states in patients with atresia of IVC.

3.5. Publication 5: Genotype-phenotype gradient of SERPINC1 variants in a single family reveals a severe compound antithrombin deficiency in a dead embryo

3.5.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Maria E de la Morena-Barrio, A Palomo, L Entrena, B de la Morena-Barrio, J Padilla, A Miñano, E Navarro, R Cifuentes, J Corral, V Vicente.
- **Journal:** British Journal of Haematology.
- **Year:** 2020.
- **Volume (Issue**): 191(1). **Pages:** e32-e35.
- **DOI:** 10.1111/bjh.16963.
- **ISSN:** 0007-1048.
- **Impact factor (JCR, 2020):** 6.998.
- **Category:** Haematology. **Position:** 12/76. Quartile 1.
- **Received:** May 24, 2020. **Accepted:** June 22, 2020.

3.5.2. Brief description

Line of research 3 was addressed in this work. We identified within our cohort of patients with antithrombin deficiency a family with strong history of embryo-foetal and neonatal deaths. Plasma antithrombin was analyzed by functional and immunological methods. Sanger sequencing of SERPINC1 was performed in peripheral blood mononuclear cells from the proband and her relatives, as well as in donated tissue from

an early miscarriage. Molecular characterization revealed two different mutations co-existing within the family: an intronic variant (c.1154-14G>A), carried by the proband, which caused a severe type I deficiency; and a Budapest 3 variant (p.Leu131Phe), carried in heterozygosis by the proband's second partner. Characterization of this family revealed an excellent genotype–phenotype gradient that covered the whole antithrombin deficiency continuum. Moreover, molecular characterization of the second dead embryo, the only available for analysis, revealed a compound heterozygosis for the type I and the type II/HBS variants, which may have led to extremely severe antithrombin deficiency and embryonic lethality. We provide new evidence in humans that a severe antithrombin deficiency might cause embryonic lethality and highlight the role of antithrombin in embryonic development.

3.6. Publication 6: A pilot study on the impact of congenital thrombophilia in COVID-19

3.6.1. Publication data

- **Authors:** Maria Eugenia de la Morena-Barrio #, Carlos Bravo-Pérez #, Belen de la Morena-Barrio, Christelle Orlando, Rosa Cifuentes, Jose Padilla, Antonia Miñano, Sonia Herrero, Shally Marcellini, Nuria Revilla, Enrique Bernal, Jose Miguel Gómez-Verdú, Kristin Jochmans, María Teresa Herranz, Vicente Vicente, Javier Corral, María Luisa Lozano (# indicates that these authors contributed equally to this work).
- **Journal:** European Journal of Clinical Investigation.
- **Year:** 2021.
- **Volume (Issue**): 51(5). **Pages:** e135-46.
- **DOI:** 10.1111/eci.13546.
- **ISSN:** 0014-2972.

- **Impact factor (JCR, 2020):** 4.686.
- **Category:** Medicine, General & Internal, Position: 35/167. Quartile 1.
- **Received:** Feb 18, 2021. **Accepted:** March 16, 2021.

3.6.2. Brief description

Line of research 4 was addressed in this study. We analyzed the clinical and biological profiles of patients with congenital thrombophilia who suffered from SARS-CoV-2 infection. Two designs were followed to identify these patients: 1) retrospective analysis of SARS-CoV-2 infection in 331 patients with prior diagnosis of congenital antithrombin deficiency from our cohort; and 2) whole exome sequencing (WES) of 87 patients below 75 years of age hospitalized with COVID-19. Overall, 15 patients with thrombophilia and SARS-CoV-2 infection were identified: four patients with prothrombotic polymorphisms (FV Leiden, N = 2; prothrombin G20210A, N = 2) and 11 patients with variants involving the genes of antithrombin (N = 6), protein S (N = 3) and protein C (N = 2). Increased D-dimer levels were found in patients with severe thrombophilia (deficiency of antithrombin, protein C or protein S) and COVID-19 compared to patients without. Patients with thrombophilia who had suffered from previous thrombotic events (N = 5), all on anticoagulant therapy at the time of SARS-CoV-2 infection, did not develop a symptomatic thrombosis during COVID-19. In contrast, one newly identified protein C-deficient patient, without thrombotic history, had VTE two days after hospital admission despite prophylaxis with heparin. This is the first study evaluating the role of congenital thrombophilia in COVID-19. Our findings suggest that severe thrombophilia may associate with a more accentuated hypercoagulable state during SARS-CoV-2 infection, and highlight the relevance of deciphering the optimal mode of thromboprophylaxis in these cases.

Chapter 4
General discussion and future perspectives

4. General discussion and future perspectives

Anti- is a Latin prefix which means against. Antithrombin is a major anticoagulant protein, and its deficiency constitutes, so far, the most severe type of inherited thrombophilia.[5] However, antithrombin has been shown to be more than a single thrombin inhibitor: it is a key molecule playing a myriad of roles, and emerging new functions of this protein have been discovered.[9]

The aim of this research was to gain further insight into antithrombin and its deficiency. Since the first report of a family with antithrombin deficiency in 1965 by Egeberg,[8] the identification and characterization of hundreds of mutant variants of antithrombin has generated a plenty of both biochemical and genetic knowledge about this and other serpins.[9] Then, considering the tremendous advance achieved in this field, the terms antithrombin and new might not seem so easy to combine any more. Nevertheless, patients constantly show us that antithrombin deficiency still encloses many puzzling questions, and some of them may even transcend thrombosis. Consequently, to reach our aim, we focused on cases with antithrombin deficiency and atypical manifestations, not previously associated to this prothrombotic disease, or emergent health problems, such as SARS-CoV-2 infection, in which the impact of inherited thrombophilias was not known. This original approach has been concretized in four specific objectives.

In line of research 1, starting from cases with unusual laboratory findings, we identified and characterized cases with transient antithrombin deficiency, that is, alternating normal and defective antithrombin levels by functional methods. The diagnostic algorithm of antithrombin deficiency follows the classic functional-biochemical-genetic sequence. Then, molecular testing is usually restricted to cases in which low antithrombin values are consistently evidenced.[4] However, this approach has the limitations of current diagnostic functional methods to detect some type II variants.

Moreover, it could potentially ignore clinically relevant cases of antithrombin, but with fluctuating expression.[9,54] Certainly, our original study design has enabled us to show that antithrombin deficiency is probably underestimated.

Although we were not able to provide an estimated prevalence of transient antithrombin deficiency, due to our methodological approach, we found that a significant proportion of cases with transient antithrombin deficiency (up to 40%) had molecular defects. Consequently, our results suggest that cases with transient deficiency might also be screened by molecular methods in selected cases, particularly when false or acquired deficiencies can be excluded. Interestingly, near 20% of cases with transient deficiency carried two recurrent SERPINC1 variants: antithrombin Cambridge II (p.Ala416Ser) and antithrombin Dublin (p.Val30Glu). Our research reveals new evidence on the thrombotic risk of these two relatively frequent variants (Minor Allele Frequency [MAF] of 0.001, approximately),[58,126,127] and encourages future studies to explore the utility of adding their genetic assessment as part of the standard of thrombophilia screening in clinical practice.

Two main different mechanisms explained transient antithrombin deficiency. Firstly, as expected, the limitation of current functional assays to detect some type II variants. Secondly, as we proposed, the combination of a molecular defect with a stress factor, which may act as a second hit to exacerbate the defective function, conformation and/ or glycosylation of antithrombin. Finally, comparison of subjects with transient and constitutive deficiency demonstrated that they not only had distinct molecular features, but also clinical ones. Thrombosis associated to transient deficiency had later onset and a lower risk of recurrence than in constitutive deficiency. We think that these findings are plausible, because all the SERPINC1 defects associated to transient deficiency were mild, missense variants. Furthermore, the deleterious consequences of some of these

variants might be exacerbated by a second, environmental factor, and this fact may necessarily result in a lower lifetime risk of thrombosis. Interestingly, the rate of arterial thrombotic events was significantly higher in these cases than in constitutive deficiency. Despite arterial thrombosis is not characteristic of antithrombin deficiency, several cases have been reported, especially in carriers of mild, type II/HBS variants.[33,98] Altogether, these facts and our results support the hypothesis that anticoagulant treatment, initiated once a VTE occurs, might subsequently mask the real risk of late-onset, arterial thrombotic events in the more severe cases. Future prospective works are required to validate these suppositions.

Overall, the work performed in line of research 1 suggests that it is probably high time to change the diagnostic algorithm of antithrombin deficiency, which, in near future, will start with genetic analysis, and will be complemented by functional and biochemical studies. However, clinical data, particularly on thrombotic events, obtained from the index cases and their relatives are crucial for family screening and clinical management. Furthermore, our findings provided more evidence in favour on the role of N-glycosylation defects (and, potentially, other post-translational modifications) in antithrombin deficiency. But the most breaking concept of this piece of work is that our results change the paradigm of congenital thrombophilia, as the pathogenic effect of a mutation might depend on external factors and could be present only at certain time points.

As opposed to the subtlety observed in cases with transient antithrombin deficiency, in lines of research 2 and 3, the association of severe antithrombin deficiency, caused by homozygous or compound heterozygosis for SERPINC1 variants, with vascular defects and embryonic lethality provided novel and strong clinical evidence supporting the impact of antithrombin deficiency in early human development. Although antithrombin

deficiency is considered the most severe thrombophilia, it certainly constitutes a highly heterogeneous disorder. The best example is antithrombin Budapest 3 variant (p.Leu131Phe), which is compatible with nearly normal function in progressive activity assays and has been shown to have null effect in the β-glycoform.[128] These observations explain the mild risk of thrombosis associated to this variant in heterozygous state, as well as the existence of viable homozygous subjects.[9,99,128]

In line of research 2, we demonstrated a high prevalence of IVC system atresia in patients homozygous for the antithrombin Budapest 3 variant. Although malformations of the IVC system were first described in 1793,[129] more than 200 years ago the molecular basis and mechanisms of this congenital anomaly have been elusive so far. The identification of IVC system atresia in up to 70% of patients homozygous for antithrombin Budapest 3 supports an important role for this genetic defect in this vascular malformation. The severe hypercoagulable state of homozygous carriers of this type II/HBS variant, together with the piece of evidence pointing to the hypothesis of in utero thrombosis,[116–120] led us to speculate that a severely impaired control of thrombin generation in Budapest 3 homozygous embryos might favor an early thrombotic event damaging the immature IVC. If not lethal, this thrombotic episode might cause vein atresia, and potentially renal agenesis. Isolated case reports of patients with IVC atresia and deficiency of antithrombin[130–132] or even deficiency of other natural anticoagulants, such as protein C[133] or protein S,[134] strongly support the association found in this study and encourages the search for congenital thrombophilia in cases with IVC atresia.

This piece of work also attracts attention to the high thrombotic risk of subjects with IVC atresia.[121] Certainly, the increased thrombotic risk of these patients may be secondary to the blood stasis induced by the absence of IVC; but if our hypothesis is confirmed by future studies, the co-existence of a severe thrombophilia in some of these

cases might identify a high-risk subgroup. Thus, the benefits of prophylactic antithrombotic procedures under risk situations might be expected in these patients. Finally, as heparin is not effective in patients with the Budapest 3 variant in homozygous state, the use of other antithrombotic treatments, such as antithrombin concentrates,[135] might be considered.

Complete absence of antithrombin causes embryonic lethality in animal models.[51,136] Aside from antithrombin Budapest 3, few additional type II variants, such as antithrombin Toyama (p.Arg79Cys), another type II/HBS variant,[9] and p.Phe261Leu, a type II/PE variant,[137] have been described in homozygous state. In line of research 3, the characterization of a dead embryo from a singular, clinical and molecularly diverse family with a strong history of embryo-foetal and neonatal deaths revealed that the miscarriage was compound heterozygote for a type I variant and antithrombin Budapest 3. Exceptionally, only three viable cases of combined type I and type II/HBS variants have been reported worldwide in two families, all again with early and life-threatening events.[138] However, no patient with type I variants in homozygous state has been described so far, suggesting, as observed in animal models, the 100% of embryonic lethality associated to this condition. Unfortunately, we were not able to determine antithrombin levels in the dead embryo; but, based on previous reports, we can speculate that the combination of these type I and type II/HBS variants may lead to an extremely severe deficiency. Anyway, this piece of work provides new evidence in humans that extremely severe antithrombin deficiency may cause embryonic lethality. The potential functions of antithrombin during the embryonic state, which may be also beyond haemostasis, should be further characterized.

Future studies are required to validate the association between severe thrombophilia and congenital malformations or embryonic lethality in humans. Animal models might

certainly be an option at the mechanistic level. They have already contributed to reveal the deleterious effect of complete antithrombin deficiency during embryonic development.[51,136,139] Illustratively, antithrombin knockout (antithrombin$^{-/-}$) mice die at 16.5 gestational days because of intravascular coagulation;[51] and targeted disruption of SERPINC1 locus in zebrafish induces lethal intracardiac thrombosis in early adulthood.[136] Nevertheless, we should be aware that animal models do not always simulate human condition. Interestingly, antithrombin$^{+/-}$ mice, resembling heterozygous type I deficiency, do not develop idiopathic thrombosis along their life.[51] Moreover, homozygous state for antithrombin Toyama in mouse models has lethal consequences; no apparent vascular malformations have seemed to be appreciated in these animals, and however they do have other abnormalities not reported in humans with severe thrombophilia, such as portal hypertension, enlarged spleen or severe eye degeneration.[139] Altogether, these observations suggest that animal models should not overpass or be estimated over clinical and translational research. Ideally, for any complex problem in Medicine, basic and clinical findings must be connected or integrated to provide the whole picture.

To further assess the impact of congenital thrombophilia in vascular malformations, we consider that the best way would be through the systematic screening of inherited thrombophilias among subjects with IVC atresia. In this sense, our group is leading a multicenter clinical and molecular study of patients with this vascular malformation, focused on the identification of thrombophilic defects.[140] By conducting WES in these patients, we aim to identify the molecular basis of IVC atresia, and to verify that it is caused by a hypercoagulable state in a significant proportion of cases. As a secondary objective, we intend to identify new forms and mechanisms of thrombophilia. This study is still in its initial phase, but as of yet, we have recruited a cohort of 170 subjects

with IVC atresia, and 50 of them with donated blood samples for WES (unpublished data). Clinical and molecular characterization of this study cohort might help us to validate the hypothesis generated by this thesis.

So far within this work, in lines of research 1 to 3, an in-depth study of antithrombin deficiency in three atypical scenarios reflect that antithrombin deficiency continuum, that is, the clinical gradient that may be found among carriers of different types and combinations of SERPINC1 defects, is wider than expected. The present research significantly supports this sentence by expanding the spectrum of antithrombin deficiency at both ends. On the mild end, we contribute by the incorporation of transient antithrombin deficiency. These cases carry mild, missense variants in SERPINC1 or hypoglycosylation defects, are elusive to functional laboratory methods and are mostly asymptomatic, but however can transiently develop late-onset, venous and arterial thrombotic events, particularly if aggravated by external factors. On the severe end, we better characterize cases with very severe homozygous type II/HBS and extremely severe compound type I/ II deficiencies, who might have a high risk of suffering from vascular malformations and even, embryonic lethality, probably as the result of in utero or perinatal thrombosis, which also gives new evidences on the key role of thrombin and its control during human embryogenesis (Figure 6).

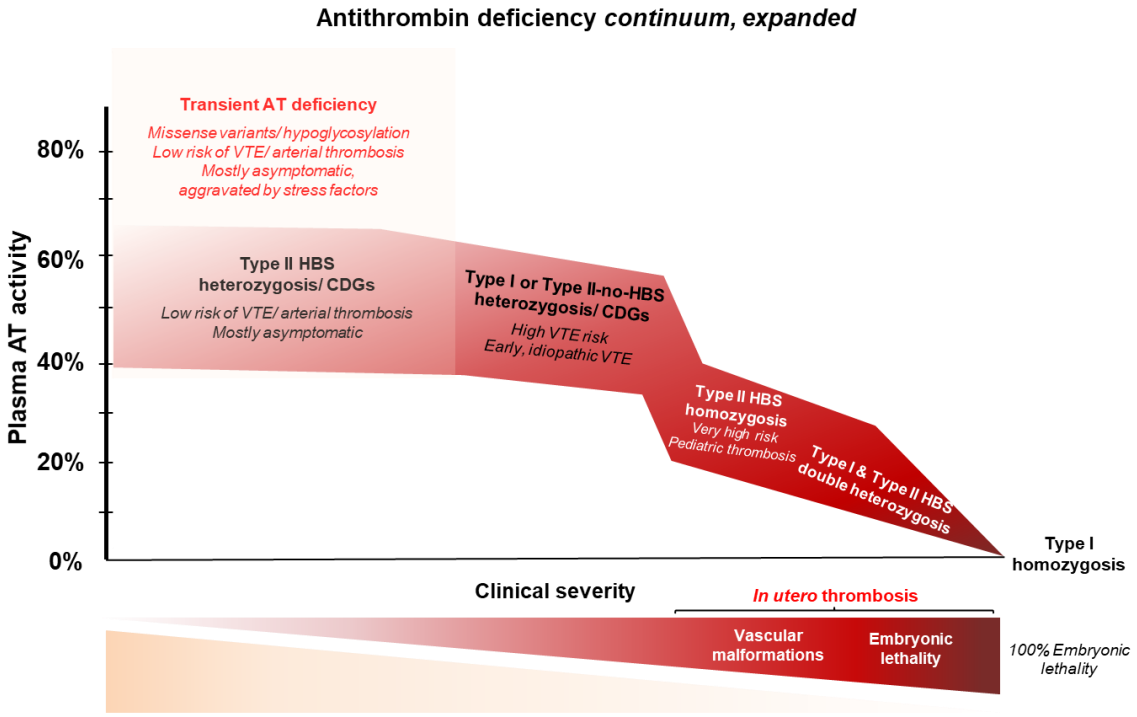

Figure 6. Antithrombin deficiency continuum, expanded. The gradient that may be found among carriers of different types and combinations of SERPINC1 defects is represented according to plasma antithrombin activity, clinical severity, and the influence of additional thrombotic factors. The frequency of cases in the general population is inverse to the clinical severity. The contributions of this thesis on expanding the continuum, particularly by introducing transient antithrombin deficiency, as well as malformations or embryonic lethality in cases with very severe antithrombin deficiency, that may be associated to in utero thrombosis, are highlighted in red. This figure is adapted from: Bravo-Perez et al. Br J Haematol. 2020, 191, e32–e34.

AT: antithrombin. CDGs: congenital disorders of glycosylation. Type II HBS: Type II heparin binding site deficiency. Type II no-HBS: Type II reactive site and pleiotropic effect deficiencies. VTE: venous

Finally, when the SARS-CoV-2 pandemic emerged, in line of research 4 we investigated the association between inherited thrombophilias and COVID-19. There are many examples of the interaction of genetic and environmental factors on the haemostatic system.[123] The explanation for the strong tendency for thrombosis in COVID-19 patients is certainly not single-factored. In this context, it seemed interesting to investigate the contribution of congenital thrombophilia.

This was the first study on the role of congenital thrombophilia in SARS-CoV-2 infection. By using two different approaches, we identified a relatively high number of patients with both mild and severe thrombophilia who suffered from COVID-19. Our analysis revealed higher D-Dimer levels in the presence than in the absence of inherited thrombophilia. The plausibility of this finding might be supported by the well-known fact that subjects with congenital thrombophilia have an impaired control of thrombin generation that may be exacerbated by risk factors.[141] The main limitation of this piece of work was the small sample size, precluding additional strong conclusions. However, it is remarkable that one of the two patients who developed a thrombotic event was identified as a carrier of protein C deficiency by WES. It is also notable that most patients with severe thrombophilia from our cohort did not develop symptomatic thrombotic events during COVID-19, including five subjects who had prior history of thrombosis. Although more evidence is required, the reason why the hypercoagulable state did not facilitate the development of new thrombotic episodes during COVID-19 might be related to the fact that these patients were already treated with anticoagulant drugs before the infection. In this sense, previous findings support that chronic anticoagulation at admission appears to protect COVID-19 patients from thrombosis.[122]

Further studies on the search of thrombophilic defects in larger cohorts would relevantly increase the statistical power to clarify the hypothesis of our pilot study. Recently, this premise has been ratified by a genome-wide association study (GWAS) in a prospective population-based cohort of 13,712 adults with COVID-19 from the UK Biobank.[142] Overall, there were 197 (1.4%) thrombotic events and 890 (6.5%) deaths in this cohort. Six polymorphisms, including FV Leiden and Prothrombin G20210A, in addition to two polygenic risk scores were evaluated. All of the aforementioned thrombophilic defects were associated to an increased risk of COVID-19 associated thrombosis, but not to

mortalily.[142] Altogether, these and our findings demonstrate that severe thrombophilia might increase the risk of thrombosis in COVID-19 by means of an exacerbated hypercoagulable state. These results suggest, as expected, that patients with inherited thrombophilia might represent a high-risk subgroup for COVID-19-associated coagulopathy and thrombosis, and for whom antithrombotic therapy should be individualized.

Chapter 5
Conclusions

5. Conclusions

5.1. Line of research 1

- Antithrombin deficiency is probably an underestimated disorder, as in our cohort we have identified cases with transient deficiency.
- A significant proportion of cases with transient antithrombin deficiency have molecular defects: SERPINC1 missense variants, two of them recurrent (antithrombin Cambridge II and antithrombin Dublin), and N-glycosylation defects.
- Cases with transient antithrombin deficiency might be screened by molecular methods in selected cases.
- Two different mechanisms mainly explain transient deficiency: the limitation of current functional assays and the combination of a genetic variant with a stress factor, impairing antithrombin function, conformation and/ or glycosylation.
- These findings change the paradigm of congenital thrombophilia, as the pathogenic effect of a mutation might depend on external factors and could be present only at certain time points.

5.2. Line of research 2

- Patients with antithrombin Budapest 3 variant in homozygous state have a high prevalence of inferior vena cava system atresia.
- This is the first evidence on the association of a genetic defect, and a severe thrombophilia, with inferior vena cava atresia, and supports that an intrauterine or perinatal thrombosis may be the underlying pathogenic mechanism.
- In a subgroup of patients with inferior vena cava atresia, the hypercoagulability conferred by the underlying thrombophilic state might contribute, together with venous stasis, to the high risk of deep venous thrombosis observed in these patients.

- Our hypothesis-generating results encourage further studies to investigate severe thrombophilic states in patients with inferior vena cava atresia.

5.3. Line of research 3

- Molecular characterization of a dead embryo from a thrombophilic family with antithrombin deficiency revealed a double heterozygosis for a quantitative and qualitative heparin-binding site SERPINC1 variants, which may have led to extremely severe antithrombin deficiency and embryonic lethality.
- This is new evidence in humans that a severe antithrombin deficiency might cause embryonic death and highlight the role of the thrombin/antithrombin axis in human development.

5.4. Line of research 4

- Severe inherited thrombophilias further accentuate the prothrombotic state in SARS-CoV-2 infection, and probably COVID-19-associated-coagulopathy and thrombosis.
- This is the first study addressing the role of congenital thrombophilia in SARS-CoV-2 infection. Further research is encouraged to clarify the consequences of inherited thrombophilic states in COVID-19, as well as to guide the optimal approach to manage anticoagulation in these cases.

5.5. Concluding remarks

In summary, despite methodological refinements or alternative study designs are needed, as previously discussed, our translational work, focused on antithrombin deficiency in atypical or emergent clinical scenarios, provides breaking concepts that confirms that, more than 50 years later, we are far from fully understanding antithrombin and its deficiency. New functions of this enigmatic glycoprotein, not only in haemostasis, continue been shown. Our work demonstrates that antithrombin

deficiency is underestimated and might be even more heterogeneous than expected. We have expanded the clinical and molecular continuum of antithrombin deficiency: from the surreptitious behaviour of transient deficiency to vascular malformations and embryonic death in the most extreme cases with compound SERPINC1 defects. Novel forms and mechanisms of antithrombin deficiency have been identified, involving other genetic loci rather than SERPINC1, such as those participating in N-glycosylation pathways. Finally, SARS-CoV-2 infection has been confirmed as an emergent prothrombotic trigger for whom patients with this and other inherited thrombophilias may be a high-risk population. Altogether, this work highlights the importance of the molecular analysis of antithrombin deficiency, not only for a better understanding of thrombosis biology, but also in terms of clinical implications.

Chapter 6
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6. References

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Chapter 7
Compendium of publications

7. Compendium of publications

7.1. Publication 1: Management of antithrombin deficiency: an update for clinicians

7.1.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Vicente Vicente, Javier Corral.
- **Journal:** Expert Review of Hematology.
- **Year:** 2019.
- **Volume (Issue):** 12 (6). **Pages:** 397-405.
- **DOI:** 10.1080/17474086.2019.1611424.
- **ISSN:** 1747-4086.
- **Impact factor (JCR, 2019):** 2.573.
- **Category:** Haematology. **Position:** 42/76. Quartile 3.
- **Received:** 19 Feb 2019. **Accepted:** 23 Apr 2019.

7.1.2. Summary

Introduction. Antithrombin is a serpin that inhibits multiple procoagulant serine proteases and acts as an endogenous anticoagulant. Thus, congenital antithrombin deficiency constitutes a major thrombophilic state, the most severe so far.

Areas covered. In the present work, we globally review the biology, genetics, diagnosis, and management of congenital antithrombin deficiency, and also discuss puzzling questions and future perspectives regarding this severe inherited thrombophilia.

Expert opinion. Although this disorder exerts high clinical heterogeneity, many carriers will need careful and long-term anticoagulation and/or thromboprophylaxis, especially in high-risk situations, such as surgery and pregnancy. Notably, antithrombin concentrates constitute a considerable arsenal for both treatment and prevention of acute

venous thrombosis in subjects with antithrombin deficiency. Current evidences are based almost exclusively on retrospective case series, so an integrated functional, biochemical and molecular characterization will be of clinical relevance and guide hematologists' personalized decisions.

7.1.3. Doctorate student contribution

As the first author of this review, the doctorate student performed extensive bibliographic search, integrated this information with his own investigations, created the tables and figures and wrote and revised the manuscript draft and its later amendments.

7.1.4. Publication URL

https://www.tandfonline.com/doi/abs/10.1080/17474086.2019.1611424?journalCode=ierr20

7.2. Publication 2: Antithrombin deficiency as a still underdiagnosed thrombophilia: a primer for internists

7.2.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Maria E de la Morena-Barrio #, Vicente Vicente #, Javier Corral # (# indicates that these authors contributed equally to this work).
- **Journal:** Polish Archives of Internal Medicine.
- **Year:** 2020.
- **Volume (Issue):** 130 (10). **Pages:** 868-877**.**
- **DOI:** 10.20452/pamw.15371.
- **ISSN:** 0032-3772.
- **Impact factor (JCR, 2020):** 3.277.
- **Category:** Medicine, General & Internal. **Position:** 54/167. Quartile 2.
- **Received:** April 8, 2020. **Accepted:** April 29, 2020.

7.2.2. Summary

Antithrombin is a key endogenous anticoagulant that also plays other roles in inflammation, immunity, and other processes. Congenital antithrombin deficiency is the most severe type of thrombophilia, yet characterized by a remarkable clinical heterogeneity. Here, as a primer for internists, we present a practical review of data regarding this disorder, focused on its molecular basis, diagnostic procedures, prognostic implications, and clinical management of patients suffering from this severe, and probably underdiagnosed, type of thrombophilia.

7.2.3. Doctorate student contribution

As the first author of this review, the doctorate student performed extensive bibliographic search, integrated this information with his own investigations, created the tables and figures and wrote and revised the manuscript draft and its later amendments.

7.2.4. Publication URL

https://www.mp.pl/paim/issue/article/15371/

7.3. Publication 3: Molecular and clinical characterization of transient antithrombin deficiency: A new concept in congenital thrombophilia

7.3.1. Publication data

- **Authors:** Carlos Bravo-Pérez, María Eugenia de la Morena-Barrio, Belén de la Morena-Barrio, Antonia Miñano, José Padilla, Rosa Cifuentes, Pedro Garrido, Vicente Vicente, Javier Corral.
- **Journal:** American Journal of Hematology.
- **Year:** 2022.
- **Volume (Issue), Pages:** 97(2):216-225.
- **DOI:** 10.1002/ajh.26413.
- **ISSN:** 0361-8609.
- **Impact factor (JCR, 2020):** 10.047.
- **Category:** Haematology. **Position:** 7/76. Decile 1.
- **Received:** November 9, 2021. **Accepted:** November 16, 2021.

7.3.2. Summary

Antithrombin deficiency, the most severe thrombophilia, might be underestimated, since it is only investigated in cases with consistent functional deficiency or family history. We have analyzed 444 consecutive, unrelated cases, from 1998 to 2021, with functional results supporting antithrombin deficiency in at least one sample. Plasma antithrombin was evaluated by functional and biochemical methods in at least two samples. SERPINC1 gene was analyzed by sequencing and MLPA. Hypoglycosylation was studied by electrophoresis and HPLC. In 260 of 305 cases (85.2%) with constitutive deficiency (activity < 80% in all samples), a SERPINC1 (N = 250), or N-glycosylation defect (N = 10) was observed, while 45 remained undetermined. The

other 139 cases had normal antithrombin activity (≥ 80%) in at least one sample, what we called transient deficiency. Sixty-one of these cases (43.9%) had molecular defects: 48 had SERPINC1 variants, with two recurrent mutations (p.Ala416Ser [Cambridge II], N = 15; p.Val30Glu [Dublin], N = 12), and 13 hypoglycosylation. Thrombotic complications occurred in transient deficiency, but were less frequent, latter-onset, and had a higher proportion of arterial events than in constitutive deficiency. Two mechanisms explained transient deficiency: The limitation of functional methods to detect some variants and the influence of external factors on the pathogenic consequences of these mutations. Our study reveals a molecular defect in a significant proportion of cases with transient antithrombin deficiency, and changes the paradigm of thrombophilia, as the pathogenic effect of some mutations might depend on external factors and be present only at certain timepoints. Antithrombin deficiency is underestimated, and molecular screening might be appropriate in cases with fluctuating laboratory findings.

7.3.3. Doctorate student contribution

As the first author of this work, the doctorate student performed extensive bibliographic search, assisted local patients, performed experiments, analyzed data, made the figures, and wrote and revised the manuscript draft and its later amendments.

7.3.4. Publication URL

https://onlinelibrary.wiley.com/doi/10.1002/ajh.26413?af=R

7.4. Publication 4: High penetrance of inferior vena cava system atresia in severe thrombophilia caused by homozygous antithrombin Budapest 3 variant: Description of a new syndrome

7.4.1. Publication data

- **Authors:** María E de la Morena-Barrio #, Réka Gindele #, Carlos Bravo-Pérez #, Péter Ilonczai #, Isabel Zuazu, Marianna Speker, Zsolt Oláh, Juan J Rodríguez-Sevilla, Laura Entrena , Maria S Infante, Belén de la Morena-Barrio, José M García, Ágota Schlammadinger, Rosa Cifuentes-Riquelme, Asunción Mora-Casado, Antonia Miñano, Jose Padilla, Vicente Vicente, Javier Corral, Zsuzsanna Bereczky (# indicates that these authors contributed equally to this work).
- **Journal:** American Journal of Hematology.
- **Year:** 2021.
- **Volume (Issue):** 96(11). **Pages:** 1363-1373.
- **DOI:** 10.1002/ajh.26304.
- **ISSN:** 0361-8609.
- **Impact factor (JCR, 2020):** 10.047.
- **Category:** Haematology. **Position:** 7/76. Decile 1.
- **Received:** April 26, 2021. **Accepted:** July 27, 2021.

7.4.2. Summary

Atresia of inferior vena cava (IVC) is a rare congenital malformation associated with high risk of venous thrombosis that still has unknown etiology, although intrauterine IVC thrombosis has been suggested to be involved. The identification of IVC atresia in a case with early idiopathic venous thrombosis and antithrombin deficiency caused by the homozygous SERPINC1 c.391C > T variant (p.Leu131Phe; antithrombin Budapest 3) encouraged us to evaluate the role of this severe thrombophilia in this vascular

abnormality. We have done a cross-sectional study in previously identified cohorts of patients homozygous for the Budapest 3 variant (N = 61) selected from 1118 patients with congenital antithrombin deficiency identified in two different populations: Spain (N = 692) and Hungary (N = 426). Image analysis included computed tomography and phlebography. Atresia of the IVC system was observed in 17/24 cases (70.8%, 95% confidence interval [CI]: 48.9%–87.3%) homozygous for antithrombin Budapest 3 with available computed tomography (5/8 and 12/16 in the Spanish and Hungarian cohorts, respectively), 16 had an absence of infrarenal IVC and one had atresia of the left common iliac vein. All cases with vascular defects had compensatory mechanisms, azygos-hemiazygos continuation or double IVC, and seven also had other congenital anomalies. Short tandem repeat analysis supported the specific association of the IVC system atresia with SERPINC1. We show the first evidence of the association of a severe thrombophilia with IVC system atresia, supporting the possibility that a thrombosis in the developing fetal vessels is the reason for this anomaly. Our hypothesis-generating results encourage further studies to investigate severe thrombophilic states in patients with atresia of IVC.

7.4.3. Doctorate student contribution

As a co-first author, the doctorate student performed extensive bibliographic search, assisted local patients, performed experiments, analyzed data, made the tables and figures, and wrote and revised both manuscript drafts and their later amendments.

7.4.4. Publication URL

https://onlinelibrary.wiley.com/doi/10.1002/ajh.26304

7.5. Publication 5: Genotype-phenotype gradient of SERPINC1 variants in a single family reveals a severe compound antithrombin deficiency in a dead embryo

7.5.1. Publication data

- **Authors:** Carlos Bravo-Pérez, Maria E de la Morena-Barrio, A Palomo, L Entrena, B de la Morena-Barrio, J Padilla, A Miñano, E Navarro, R Cifuentes, J Corral, V Vicente.
- **Journal:** British Journal of Haematology.
- **Year:** 2020.
- **Volume (Issue**): 191(1). **Pages:** e32-e35.
- **DOI:** 10.1111/bjh.16963.
- **ISSN:** 0007-1048.
- **Impact factor (JCR, 2020):** 6.998.
- **Category:** Haematology. **Position:** 12/76. Quartile 1.
- **Received:** May 24, 2020. **Accepted:** June 22, 2020.

7.5.2. Summary

We identified within our cohort of patients with antithrombin deficiency a family with strong history of embryo-foetal and neonatal deaths. Plasma antithrombin was analyzed by functional and immunological methods. Sanger sequencing of SERPINC1 was performed in peripheral blood mononuclear cells from the proband and her relatives, as well as in donated tissue from an early miscarriage. Molecular characterization revealed two different mutations co-existing within the family: an intronic variant (c.1154-14G>A), carried by the proband, which caused a severe type I deficiency; and a Budapest 3 variant (p.Leu131Phe), carried in heterozygosis by the proband's second partner. Characterization of this family revealed an excellent genotype–phenotype

gradient that covered, and perfectly illustrated, the whole antithrombin deficiency continuum. Moreover, molecular characterization of the second dead embryo, the only available for analysis, revealed a double heterozygosis for the type I and the type II/HBS variants, which may have led to extremely severe antithrombin deficiency and embryonic lethality. We provide new evidence in humans that a severe antithrombin deficiency might cause embryonic lethality and highlighted the role of antithrombin in embryonic development.

7.5.3. Doctorate student contribution

As first author, the doctorate student performed extensive bibliographic search, performed experiments, analyzed data, made figures, and wrote and revised both manuscript drafts and their later amendments.

7.5.4. Publication URL

https://onlinelibrary.wiley.com/doi/10.1111/bjh.16963

7.6. Publication 6: A pilot study on the impact of congenital thrombophilia in COVID-19

7.6.1. Publication data

- **Authors:** Maria Eugenia de la Morena-Barrio #, Carlos Bravo-Pérez #, Belen de la Morena-Barrio, Christelle Orlando, Rosa Cifuentes, Jose Padilla, Antonia Miñano, Sonia Herrero, Shally Marcellini, Nuria Revilla, Enrique Bernal, Jose Miguel Gómez-Verdú, Kristin Jochmans, María Teresa Herranz, Vicente Vicente, Javier Corral, María Luisa Lozano (# indicates that these authors contributed equally to this work).
- **Journal:** European Journal of Clinical Investigation.
- **Year:** 2021.
- **Volume (Issue**): 51(5). **Pages:** e135-46.
- **DOI:** 10.1111/eci.13546.
- **ISSN:** 0014-2972.
- **Impact factor (JCR, 2020):** 4.686.
- **Category:** Medicine, General & Internal, Position: 35/167. Quartile 1.
- **Received:** Feb 18, 2021. **Accepted:** March 16, 2021.

7.6.2. Summary

We analyzed the clinical and biological profiles of patients with congenital thrombophilia who suffered from SARS-CoV-2 infection. Two designs were followed to identify these patients: 1) retrospective analysis of SARS-CoV-2 infection in patients with prior diagnosis of congenital antithrombin deficiency (N = 331); and 2) whole exome sequencing of 87 patients below 75 years of age hospitalized with COVID-19. Overall, 15 patients with thrombophilia and SARS-CoV-2 infection were identified:

four patients with prothrombotic polymorphisms (FV Leiden, N = 2; prothrombin G20210A, N = 2) and 11 patients with variants involving the genes of antithrombin (N = 6), protein S (N = 3) and protein C (N = 2). Increased D-dimer levels were found in patients with severe thrombophilia (deficiency of antithrombin, protein C or protein S) and COVID-19 compared to patients without. Patients with thrombophilia who had suffered from previous thrombotic events (N = 5), all on anticoagulant therapy at the time of SARS-CoV-2 infection, did not develop a symptomatic thrombosis during COVID-19. In contrast, one newly identified protein C-deficient patient, without thrombotic history, had VTE two days after hospital admission despite prophylaxis with heparin. This is the first study evaluating the role of congenital thrombophilia in COVID-19. Our findings suggest that severe thrombophilia may associate with a more accentuated hypercoagulable state during SARS-CoV-2 infection, and highlight the relevance of deciphering the optimal mode of thromboprophylaxis in these cases.

7.6.3. Doctorate student contribution

As a co-first author, the doctorate student performed extensive bibliographic search, performed experiments, analyzed data, made the tables and figures, and wrote and revised both manuscript drafts and their later amendments.

7.6.4. Publication URL

https://onlinelibrary.wiley.com/doi/10.1111/eci.13546