UNIVERSITAT DE BARCELONA

L´hemostàsia primària en la trombosi venosa. Biomolècules implicades en la formació del coàgul plaquetari: paper de VAMP8, SERT, SEROTONINA, ADAMTS13 i Factor von Willebrand

Ma. Dolors Llobet i Lorente

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Barcelona, 2020, Ma. Dolors Llobet i Lorente

Disseny de la portada: Fernan Duran i Dolors Llobet
Figures: Clara Morales Llobet
Disseny i composició: Clara Morales i Dolors Llobet

Tesi Doctoral

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Ma Dolors Llobet i Lorente

Treball realitzat per a optar al grau de Doctora en Biomedicina sota la direcció de Jordi Fontcuberta Boj i Juan Carlos Souto Andrés, a la Unitat d'Hemostàsia i Trombosi de l'Hospital de la Santa Creu i Sant Pau.

Servei d'Hematologia

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No es pot pactar amb les dificultats, les vencem o ens vencen
El Chojin

Als meus pares i a la Fernan
en memòria de l'Àlex

AGRAÏMENTS

A tots els que heu fet possible que jo vencés les meves dificultats:

A la meva família, per tot el seu suport i acompanyament en tot aquest camí, en especial als meus pares per ensenyar-me que la constància i l'esforç donen el seu fruit.

A la Fernan, per tot el seu ajut i encoratjament durant tot aquest camí i com no, per tots els retrats i dibuixos que ha fet mentre jo intentava escriure aquest llibre...gràcies per fer tant fàcil compartir la vida.

Al Sergi i al Dr. Civil per haver-me orientat en el meu creixement personal.

A la Montse Borrell, per tota la seva valuosa ajuda i suport en tots aquests treballs de recerca.

A la Cris Vallvé, per no dubtar mai a donar-me un cop de mà en el dia a dia i fer que compartir la feina amb ella sigui un plaer.

Al Jordi Fontcuberta, per la seva saviesa tant científica com personal, treballar tants anys amb ell ha estat un privilegi, gràcies també pel teu ajut i compromís en la direcció d'aquesta tesi.

Al Joan Carles Souto pel seu compromís i responsabilitat vers la direcció d'aquesta tesi.

A la Isa Tirado i a l'Artur Oliver, per totes les tardes d' "estadística"...sense ells no hauria estat possible aquesta recerca.

A la Lídia Romero per que treballar amb ella és un regal.

A la Tere Urrutia, la Rosa Felices i la Imma Coll per que els hi dec tot el que he après al laboratori.

Al Quino Murillo per que sense les seves bases de dades tampoc hauria estat possible aquest treball.

A la Noèlia Vilalta i al Jose Mateo per la seva guia en el dia a dia.

To Professor Stone, without whose wisdom these papers would not have been accepted. His view on life guides me through my daily routine. Thank you, Bill,

for treating everyone with the same amount of respect independently of their prestige.

Per últim a tots les ajuts econòmics: FIS PI12/00612, FIS PI 15/0269- RIC, RD12/0042/0032, SAF 2005-04738 i SAF 2008-08159.

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ABREVIATURES:

5-HT	Serotonina
5-HT_{2A}	Receptor de la Serotonina
5HTT	Transportador de la Serotonina
5-HTTLPR	Regió polimòrfica del gen del transportador de la serotonina
95%CI	Intèrval de confiança del 95%
ADAMTS13	a disintegrin and metalloproteinase with thrombospodin-1-like domains
ADP	Adenosin difosfat
ADP_AM	Màxima agregació induïda per adenosina difosfat
ADP_AUC	Àrea sota la corba de l'agregació plaquetària induïda per adenosina difosfat.
AGR	agregació màxima
AINES	Antiinflamatoris no esteroideus
AMPc	Adenosin monofosfat cíclic
ANOVA	Anàlisi de variança (*ANalysis Of VAriance*)
ATIII	Antitrombina III
ATP	Adenosin trifosfat
AUC	Àrea sota la corba
BIC	criteri d'informació Bayesiana (Bayesian Information Criterion)
BMI	Body mass index
BT	Temps de sagnat (*Bleeding Time)*
CD4	Cúmul de diferenciació 4 (*cluster of cuadruple differentiation)*
CD36	Glicoproteïna plaquetària 4
CD62	P-selectina
Col-ADP	Temps d'oclusió de col.lagen-adenosin difosfat
Col-EPI	Temps d'oclusió de col.lagen- epinefrina
CTs	Temps d'oclusió (*Closure times*)
DVT	Trombosi venosa profunda (*Deep Vein Thrombosis*)
ECTIM	*Etude Cas-Témoin de l'Infarctus du Myocarde*
EDTA	Àcid etlendiaminotetraacètic (*ethylene diamine tetra acetate*)
ELISA	Assaig d' inmunoabsorció lligat a enzims (*enzyme linked immunosorbent assays*)
EPI	Epinefrina
EPI_AM	Màxima agregació plaquetària induïda per epinefrina
EPI_AUC	Àrea sota la corba de l'agregació plaquetària induïda per epinefrina.
F	Dones (*Females*)
FII	Factor II
FIIa	Trombina

FIX	Factor IX
FIXa	Factor IX activat
FT	Factor Tissular
FV	Factor V
FVa	Factor V activat
FVII	Factor VII
FVIIa	Factor VII activat
FVIII	Factor VIII
FVIIIa	Factor VIII activat
FVL	Factor V Leiden
FvW	Factor von Willebrand
FX	Factor X
FXa	Factor X activat
FXI	Factor XI
FXIa	Factor XI activat
FXII	Factor XII
FXIIa	Factor XII activat
GAIT-2	*Genetic Analysis of Idiopathic Thrombophilia-2*
GP IaIIa	Receptor del col.lagen
GPCR	Receptors de proteïna G
GPIb	Glicoproteïna Ib
GPIb/IX/V	Complex glicoproteic Ib/IX/V
GPIIb/IIIa	Glicoproteïna IIb/IIIa
GPVI-FcRγ	Complex glicoproteic (GPVI)/FcRγ
GWAS	Estudi d'associació del genoma complet (genome-wide association study)
GαZ	Subunitat α- de la proteïna heteromètrica Gz
HCII	Cofactor II de l'heparina
hyper-ADP	Individus hiperreactius per l'inductor Adenosina difosfat
hyper-EPI	Individus hiperreactius per l'inductor epinefrina
IAM	Infart agut de miocardi
ICAM-1	Molècula d'adhesió intercel.lular 1 (*Intercellular Adhesion Molecule 1*)
ICAM-2	Molècula d'adhesió intercel.lular 2 (*Intercellular Adhesion Molecule 2*)
IL-8	Interleucina-8
IMC	Index de massa corporal
LTA	Agregometria de transmissió de llum (*Light Transmission Aggregometry*)
M	Homes (males)
MA	Agregació màxima
Met	Metionina
MI	Infart de miocardi

MIP1	Proteïnes inflamatòries del macròfag (*Macrophage Inflammatory Proteins*)
NET's	Trampes extracel.lulars de Neutròfils (*Neutrophil extracellular traps*)
NO	Òxid Nítric
NS	No significatiu
NSAID	*non-steroidal antiinflammatory drugs*
OR	Odds ratio
$P2Y_1$	Receptor purinèrgic $P2Y_1$
$P2Y_{12}$	Receptor plaquetar $P2Y_{12}$
PAI	Inhibidor de l'activador del plasminogen
PAR-1	Receptors de proteases activat-1
PAR-2	Receptors de proteases activat-2
PCR	Reacció en cadena de la polimerasa (*polymerase chain reaction*)
PDGF	Factor de creixement derivat de plaquetes (*platelet derived growth factor*)
PE	Embolisme pulmonar (*Pulmonary Embolism*)
PEAR-1	Receptor plaquetari d'agregació endotelial (*Platelet endothelial aggregation receptor -1*)
PECAM	Molècula d'adhesió de la cèl.lula endotelial- plaqueta (*Platelet endothelial cell adhesion molecule*)
PFA_ADP	Funcionalisme plaquetari induït per adenosin difosfat.
PFA_EPI	Funcionalisme plaquetari induït per epinefrina.
PFA-100	Analitzador de funcionalisme plaquetar-100
PFAadp	Funcionalisme plaquetar induït per adenosin difosfat.
PFAepi	Funcionalisme plaquetar induït per epinefrina.
PGI_2	Prostaciclines
PLT	Plaquetes
PPP	Plasma pobre en plaquetes
PRP	Plasma ric en plaquetes
PT20210A	Polimorfisme de la protrombina 20210A
PTT	Púrpura trombòtica trombocitopènica
QTL	Locus de un caràcter quantitatiu (*quantitative trait locus*)
RETROVE	Risc de Enfermedad TROmboembólica VEnosa
RPFA	Assaig de la suma del funcionalisme plaquetari induït per ADP i també per EPI (*redundant platelet function assay*)
SD	Desviació estàndard
SERT	Transportador de la serotonina
SLC6A4 gene	Gen del transportador de la serotonina
SLP	Màxima pendent de la corba (*maximum slope of the curve*)
SNP	Polimorfisme de nucleòtid únic (Single Nucleotide Polymorphism)

SOLAR	Programa estadístic per a genética (*Sequencial Oligogenic Linkage Analysis Routine*)
SPS	Síndrome de les plaquetes enganxoses (*Sticky Platelet Syndrome*)
SPSS	Programa estadístic per a ciències socials (Statistical Package for the Social Sciences)
SSRI	Inhibidors de la recaptació de la Serotonina (*Selective inhibition of serotonin re-uptake*)
TFPI	Inhibidor de la via del factor tissular (*Tissue Factor Pathway Inhibitor*)
TM	Trombomodulina
TMA	Trimetilamina
TMAO	Trimetilamina N-òxid
TNF	Factor de Necrosi Tumoral
t-PA	Activador tissular del plasminogen
TPα	Receptor del tromboxà α
TPβ	Receptor del tromboxà β
t-SNARE	diana- receptors de proteínes de fixació soluble de NSF (target-*Soluble NSF Attachment Protein Receptors)*
TXA_2	Tromboxà
TXA_2R	Receptor del Tromboxà
Tyr	Tirosina
uPAR	Receptors per l'activador del plasminogen tipus uroquinasa
VAMP3	Proteïna de membrana 3 associada a vesicules (Vesicle-Associated Membrane Protein 3)
VAMP8	Proteïna humana de membrana 8 associada a vesicules (Human Vesicle-Associated Membrane Protein 8)
Va-Xa-II	Complex protrombinasa
VCAM	Molècula d'adhesió de la cèl.lula vascular 1 (*vascular cell adhesion molecule 1*)
VIIIa-IXa-X	Tenasa
v-SNARE	vesícula- receptors de proteínes de fixació soluble de NSF (*vesicle-Soluble NSF Attachment Protein Receptors)*
VT	Trombosi venosa (*venous thrombosis*)
VTE	Tromboembolisme venós (*venous thromboembolism*)
vWf	Factor von Willebrand
VWF	Factor von Willebrand
vWF	Factor von Willebrand
WB_ADP	Agregació de adenosina difosfat en sang total
WHO	Organització mundial de la salut (World Health Organitzation)
$α_2B_1$	Integrina α2B1
$α_5β_1$	Receptor de la fibronectina

$\alpha_{IIb}\beta_3$	Integrina αIIbβ3
$\alpha_v\beta_3$	Receptor de la vitronectina
$\chi 2$	Chi quadrat

PRIMARY HEMOSTASIS IN VENOUS THROMBOSIS. BIOMOLECULES INVOLVED IN THE FORMATION OF PLATELET CLOT: PAPER OF VAMP8, SERT, SEROTONIN, ADAMTS13 AND von WILLEBRAND FACTOR.

The role of platelet in bleeding has been studied widely but little is known about their role in venous thrombosis (VT). Recently, platelet hyperreactivity has been described as associated with thrombotic risk. Platelet hyperreactivity is measured by aggregation in platelet rich plasma (platelet hyper-aggregability) or through test with whole blood as PFA-100® system. Recently, our group found that platelet function (measured by PFA-100® system) was associated with VT.

The objective of my research is to evaluate the role of platelets in VT and the role of plasmatic biomolecules in primary hemostasis as *human vesicle associated membrane protein 8* (VAMP8), serotonin, *serotonin transporter* (SERT), *a disintegrin and metalloproteinase with thrombospodin-1-like domains* (ADAMTS13) and *von Willebrand factor* (vWf).

The main conclusions in the first article were that platelet hyper-aggregability is not independently associated with VT risk. The correlation between platelet hyper-aggregability and platelet function was modest (only 12% of PFA-100® values was estimated to be due to platelet aggregation).

Although, no association was found between platelet hyper-aggregability and VT, however our group found an association between platelet hyperreactivity (measured by PFA-100® system) and VT. This finding motivated us to examine platelet hyperreactivity following treatment with some biomolecules related to this phenotype and platelet aggregation as VAMP8, SERT and serotonin.

In the second article we reported that VAMP8 and SERT levels were independently associated with VT in women. We did not find a correlation between these biomolecules and PFA-100® values.

Endothelium plays an important role in primary hemostasis. Endothelial cell and platelets synthesize vWf that is necessary for platelet adhesion. ADAMTS13 is the regulator of vWf. Both are associated with arterial thrombosis but little is known about their relation with VT.

The third article reports that low levels of ADAMTS13 were associated with high VT risk in women and that vWf levels were blood group dependant while ADAMTS13 were not.

Finally, the fourth article reports that a genome-wide association study (GWAS) identified susceptibility loci for PFA-100® phenotypes. Our results suggest that *ABO* locus is the main determinant of PFA-100® phenotypes.

CAPÍTOL 1

INTRODUCCIÓ

CAPÍTOL 1

INTRODUCCIÓ:

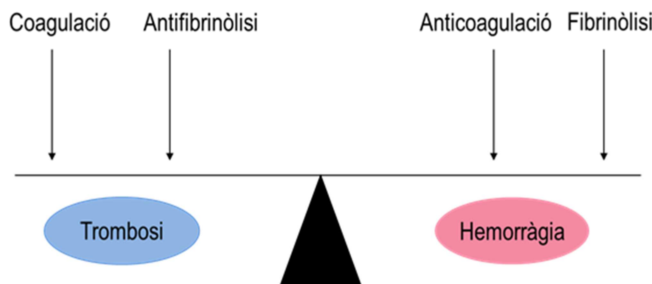
1.1. L'HEMOSTÀSIA

La sang és un teixit líquid. El manteniment d'aquest estat és el principal objectiu de l'hemostàsia [1]. Així doncs podríem definir l'hemostàsia com el conjunt de processos que eviten la pèrdua de sang quan s'ha lesionat un vas, mitjançant la formació del coàgul (estat sòlid) i que restableixen el pas de la sang quan un vas queda tapat mitjançant la destrucció del coàgul (estat líquid). El procés final de la formació del coàgul és la formació de la xarxa de fibrina (proteïna insoluble) on queden incloses plaquetes, leucòcits i eritròcits.

Les reaccions que es produeixen es poden classificar de la següent manera segons la seva funció:

- Activació de les plaquetes i formació del coàgul plaquetari (hemostàsia primària)
- Pro coagulants: condueixen a la formació de fibrina
- Anticoagulants: regulen la coagulació evitant que s'estengui de manera incontrolada
- Fibrinolítiques: eliminen la fibrina quan ja no és necessària restablint el flux sanguini
- Antifibrinolítiques: regulen la fibrinòlisi.

Quan es trenca l'equilibri entre tots aquests processos es produeix una hemorràgia o una trombosi:



La patogènesi de la trombosi va experimentar un gran progrés quan es va constatar que l'hemostàsia i la trombosi són processos que es formen en un punt concret, la zona endotelial deteriorada. Llavors la coagulació s'inicia: el col·lagen del subendoteli queda exposat a la sang i el factor tissular (FT) s'expressa, seguidament s'uneix al factor VII activat. L'exposició del col·lagen fa que les plaquetes s'uneixin al col·lagen, s'activin, es desgranulin i s'agreguin formant el primer tap hemostàtic. Simultàniament, la coagulació s'activa, per exposició del FT, resultant-ne la formació de la fibrina en el lloc on s'ha produït la lesió vascular i l'estabilització del coàgul plaquetar inicial.

La majoria dels factors de la coagulació són proteïnes que es troben a sang com a zimògens inactius i que poden ser activats i transformats en enzims amb activitat serin-proteasa mitjançant proteòlisi limitada. La majoria dels factors de la coagulació es sintetitzen al fetge i alguns d'ells necessiten la vitamina K per a la seva síntesi completa (factors vitamino K dependents: són els factors: II, VII, IX, X, la proteïna C i la proteïna S). La vitamina K actua com a cofactor permetent la carboxilació dels residus d'àcid glutàmic d'aquests factors. Aquesta transformació els hi confereix una gran afinitat pels fosfolípids. D'aquesta manera, la reacció que en fase fluida seria molt poc eficient, es realitza sobre superfícies fosfolipídiques (les membranes cel·lulars) formant complexos enzimàtics que augmenten considerablement l'eficàcia de la reacció. Es requereix també la presencia de Ca^{++} en la majoria de les reaccions.

Hi ha altres proteïnes que no tenen activitat per elles mateixes, però que actuen com a cofactors en els complexos enzimàtics, augmentant la eficiència de la reacció. Són els factors plasmàtics V i VIII que després de patir una petita proteòlisi per la trombina (IIa), formen part del complex protrombinasa (Va-Xa-II) i tenasa (VIIIa-IXa-X) respectivament. També són cofactors les proteïnes de membrana: factor tissular (FT), trombomodulina i el receptor de la proteïna C.

El mecanisme hemostàtic fisiològicament rellevant està associat a tres complexos enzimàtics procoagulants: el complex FT-VIIa, el complex protrombinasa i la tenasa. Aquests complexos augmenten la velocitat de reacció en l'activació del seu substrat unes 10^5-10^6 vegades. El fet de requerir la formació d'aquests complexes catalítics sobre la membrana cel·lular fa que la coagulació es produeixi en el lloc on s'ha lesionat el vas i no es propagui lluny d'on s'ha iniciat.

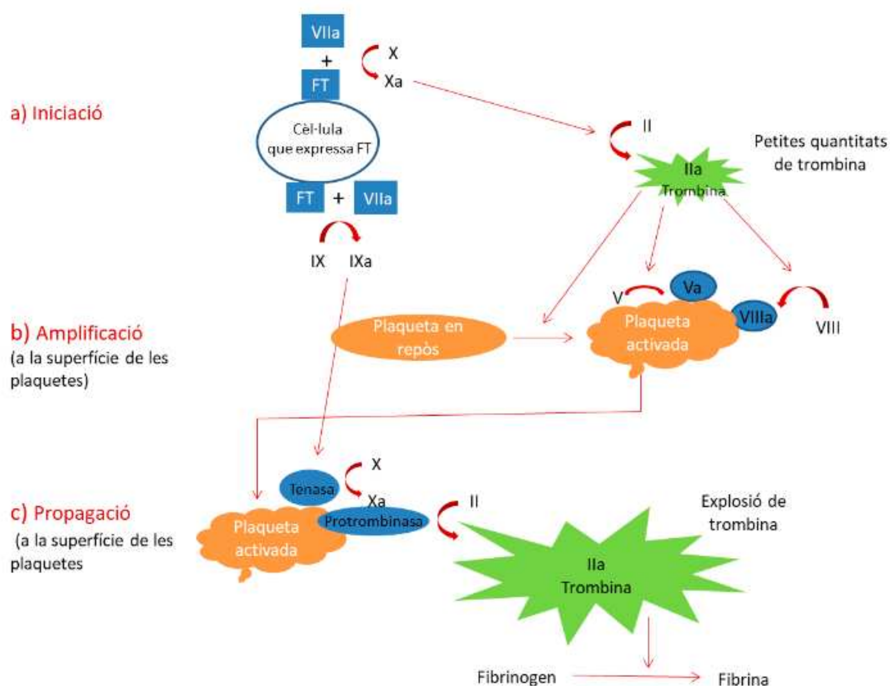
Avui en dia es considera la coagulació com un procés basat en la interacció cel·lular desestimant-se així l'antic concepte d'activació en forma de cascada (iniciat per la via intrínseca o extrínseca). Això es degut a un coneixement més gran tant del rol de cada factor de la coagulació com de les cèl·lules implicades. Aquest model cel·lular de la coagulació mostra més adequadament la interacció entre els enzims de la coagulació i les membranes d'algunes cèl·lules (plaquetes, monòcits, cèl·lules endotelials, etc) per a generar trombina i formar el coàgul.

Sembla doncs que *in vivo* l'activació de les plaquetes té un paper fonamental en la generació de trombina. Podem dividir fisiològicament la coagulació en tres fases:

- Iniciació: quan es produeix una lesió vascular el factor tissular (FT) s'uneix al FVIIa circulant. Aquest complex és capaç d'activar el FX a FXa que a la vegada és capaç de produir petites quantitats de trombina que ja poden iniciar la coagulació. El complex FT-VIIa activa també el FIX a FIXa.

- Amplificació: les petites quantitats de trombina generades activen les plaquetes promovent la seva agregació **(hemostàsia primària)** i augmenten la generació de FXa. Les plaquetes s'acumulen. La trombina activa petites quantitats de factor V i VIII (cofactors) que s'uneixen a la superfície fosfolípidica. La resposta de la coagulació s'amplifica.

- Propagació: les proteases activades interaccionen amb els seus cofactors creant-ne la tenasa i la protrombinasa. La tenasa activarà el factor X a FXa i la protrombinasa activarà la protrombina a trombina de manera molt mes eficient. Aquesta trombina transformarà el fibrinogen en fibrina, formant-se així la malla de fibrina on quedarien atrapades les plaquetes, els hematies i els leucòcits. Aquesta fibrina quedarà estabilitzada per l'acció del FXIII que prèviament ha estat activat per la trombina.



L'hemostàsia primària intervé en la trombosi arterial o venosa mitjançant dos vies: les plaquetes i l'endoteli vascular. Descriuré amb més detall la formació del tap hemostàtic o coàgul plaquetari (hemostàsia primària):

1.2. LES PLAQUETES I LA FORMACIÓ DEL COÀGUL PLAQUETARI:

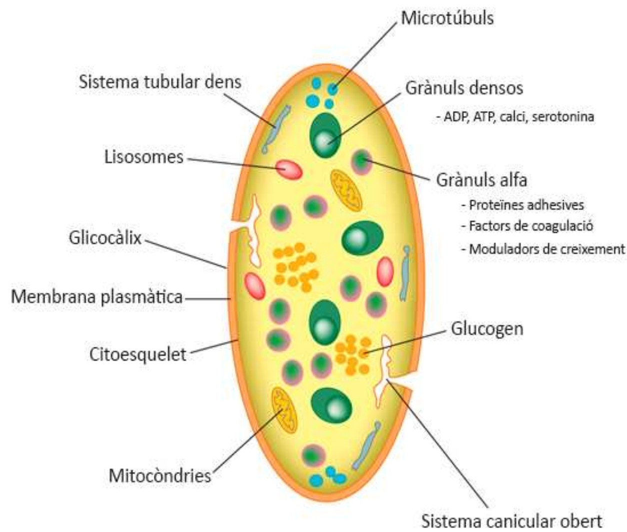
1.2.1 Estructura plaquetària:

Les plaquetes són fragments del citoplasma dels megacariòcits amb una mida de 2-4 µm. Són de forma discoide i anucleades. Tenen un paper essencial en l'hemostàsia i en la fisiopatologia de la aterotrombosi. Hi ha dos bilions de plaquetes circulant. Tenen una vida mitja de 8 a 10 dies. Es produeixen diàriament 10^5 milions de noves plaquetes dels megacariòcits del moll de l'ós per tal de mantenir el recompte normal de 150-400 $x10^9$ plaquetes per litre de sang.
La membrana de les plaquetes s'estén mitjançant el sistema canicular cap al interior del citoplasma, connectant-se així amb el citosol on trobem, lisosomes, grànuls densos (δ), grànuls α i algunes mitocòndries.

- Als grànuls α hi trobem moltes proteïnes, per exemple: factor 4 plaquetar, β-tromboglobulina, trombospondina, CD4, factor V i la glicoproteïna GPIIb/IIIa. També hi trobem factors pro-trombòtics: factor von Willebrand (FvW) i fibrinogen; proteïnes adhesives: P-selectina; factor de creixement derivat de les plaquetes (PDGF), i factors pro-inflamatoris: IL-8, MIP1.

- Els grànuls densos tenen molècules petites, són rics en nucleòtids (ADP, ATP), serotonina, Ca^{++} i polifosfats.

- Els lisosomes alliberen enzims: catepsines, hexosaminidasa



Fuente: Jesús A. Fernández-Tresguerres: *Fisiología humana*, 4e: www.accessmedicina.com
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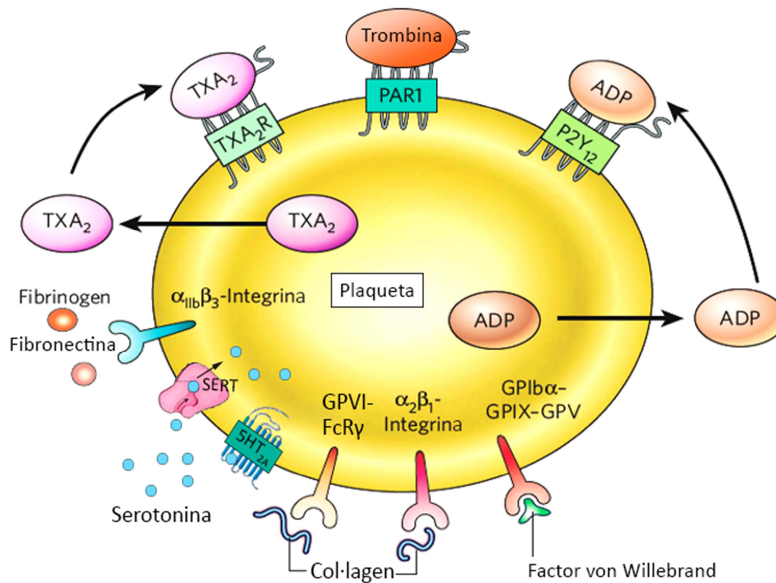
Hi ha una gran varietat de receptors transmembrana que composen la membrana de la plaqueta:

- Receptors de proteïna G (GPCR):
- Receptors de trombina: PAR-1 y PAR-2
- Receptors de ADP: P2Y1 i P2Y12
- Receptors del tromboxà: TPα i TPβ
- Receptors de Serotonina: $5\text{-}HT_{2A}$
- Transportador de la Serotonina (SERT) (5HTT).

- Integrines: $\alpha_{IIb}\beta_3$, $\alpha_2 B_1$.
- Glicoproteïnes (amb dominis rics en leucina): GPIb/IX/V i receptors Toll-like.
- Receptors de lecitina tipus C: P-selectina
- Proteïnes que provenen de la superfamília d'immunoglobulines: GPVI-FcRγ
- Una miscel·lània d'altres tipus de receptors: CD36, lligand 1 de P-selectina, receptor tipus TNF.



Aquests receptors es troben en diferents cèl·lules però alguns són específics de les plaquetes on tenen un paper fonamental en l'hemostàsia i en la cèl·lula endotelial.

Un cop activades, les plaquetes canvien la seva forma, de discoide a esfèrica i emeten pseudopodis per tal d'augmentar la superfície de contacte. Quan la plaqueta s'ha activat, s'allibera el contingut dels grànuls per a promoure l'adhesió plaquetària i l'agregació en la zona danyada de l'endoteli vascular.

1.2.2 Formació del coàgul plaquetari:

Depèn de l'acció coordinada dels següents processos 1) fixació de les plaquetes a l'endoteli alterat (fase de iniciació), 2) emmagatzement i activació de les plaquetes (fase d'extensió) i 3) estabilització del tap plaquetari i prevenció de la desagregació (fase d'estabilització).

1) FASE D'INICIACIÓ:

Les plaquetes circulen en condicions normals en la perifèria del flux sanguini, de manera que augmenta la seva disponibilitat a prop de la paret vascular. Quan es produeix una lesió en la paret vascular, el col·lagen del subendoteli queda exposat, això afavoreix al FvW,que es troba al plasma i a la paret vascular, que s'hi uneixi. Quan s'altera l'endoteli ràpidament succeeix una vasoconstricció. En les venes i arteries grans hi ha forces de cisallament baixes, llavors l'adhesió de les plaquetes a l'endoteli vascular alterat es produeix mitjançant el col·lagen. En les petites arteries hi ha forces de cisallament altes, llavors l'adhesió plaquetar depèn de la unió al FvW. Les plaquetes mitjançant el receptor GPIb s'uneixen al FvW. D'aquesta manera queden adherides a la paret vascular formant una monocapa (**adhesió plaquetària**)

La deficiència del receptor GPIb està associada a la síndrome de Bernard-Soulier que es caracteritza per les plaquetes gegants i una diàtesi hemorràgica moderada.

Aquesta unió promou la captació de més plaquetes a la zona endotelial alterada i la formació d'unions més estables de les plaquetes, s'inicia llavors l'activació plaquetària. S'ha demostrat que la unió de la GPIb al FvW immobilitzat dóna lloc a un increment de Ca^{++} citoplasmàtic, fosforilació de proteïnes, alliberament d' ADP, síntesi de tromboxà A_2 (TxA_2) i agregació plaquetària.

2) FASE D'EXTENSIÓ:

A partir d'aquí es produeix l'**activació plaquetària** mitjançant productes de secreció com l'ADP, el TxA_2, l'epinefrina, o la trombina i aquesta activació provocarà un canvi conformacional en una glicoproteïna de membrana

plaquetar (GP IIb-IIIa) que permetrà la unió entre elles mitjançant el fibrinogen. L'activació plaquetària provoca també canvis de forma de les plaquetes passant de discoïdals a esfèriques i a desenvolupar pseudopodis. Aquesta unió interplaquetar s'anomena **agregació plaquetària**.

Rol dels inductors alliberats de les plaquetes:

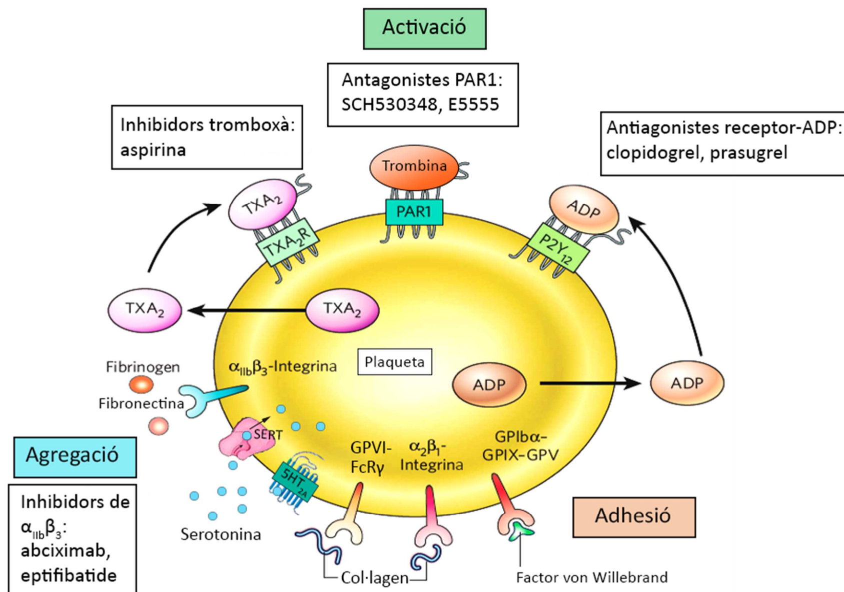
Tromboxà A_2 (TxA_2) Se sintetitza en les plaquetes activades mitjançant una sèrie de reaccions de la ciclooxigenasa i la tromboxà sintetasa A_2. És un derivat de l'àcid araquidònic (que s'obté mitjançant la dieta o bé per la conversió de l'àcid linoleic). La ciclooxigenasa-1 dóna lloc a derivats de l'àcid araquidònic com les prostaglandines, les prostaciclines i el tromboxà.
El TxA_2 és un dels agents pro agregants més potents i també un gran vasoconstrictor. El seu excés s'associa a fenòmens trombòtics. La síntesi de tromboxà es bloqueja amb l'àcid acetilsalicílic (aspirina) mitjançant el bloqueig de la ciclooxigenasa, en concret acetila i inactiva un residu de serina del centre actiu de la ciclooxigenasa.

Trombina: Es l'enzim clau de la coagulació: com ja s'ha explicat anteriorment es genera en l'endoteli danyat i té un paper fonamental en l'estabilització del coàgul plaquetari mitjançant la formació de malles de fibrina. La trombina, mitjançant la via dels receptors de proteases activats (PAR), fonamentalment pel PAR-1 i el PAR-4, és l'activador més efectiu de les plaquetes: n'indueix canvis de forma, síntesi i secreció de tromboxà, mobilització del Ca^{++}, fosforilació de proteïnes i agregació plaquetària.

Adenosina difosfat (ADP) S'allibera dels grànuls densos de les plaquetes i també dels eritròcits. Mitjançant l'augment del Ca^{++} intraplaquetar, la fosforilació de proteïnes i la síntesi de tromboxà realitza l'agregació plaquetària. Aquests processos es realitzen a través de la interacció de l'ADP amb dos quimioreceptors del grup de receptors de proteïna G: el $P2Y_1$ i el $P2Y_{12}$. Ela fàrmacs antiagregants clopidogrel, prasugrel i altres tienopiridines tenen com a diana el receptor $P2Y_{12}$. La deficiència congènita d'aquest receptor cursa amb una diàtesi hemorràgica moderada.

Epinefrina (EPI) o adrenalina: La epinefrina, tant la secretada localment des de les plaquetes activades com la circulant, contribueix al creixement del coàgul

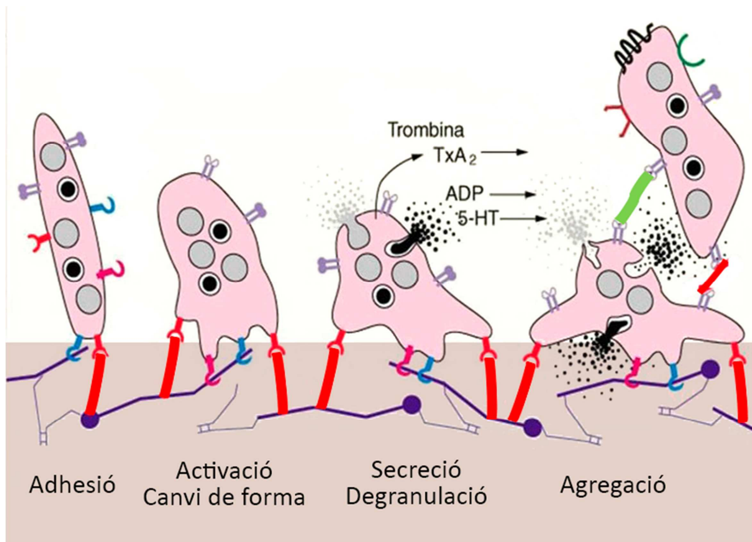
plaquetari. És un dèbil inductor de les plaquetes però quan actua sinèrgicament amb altres inductors, encara que sigui a baixes concentracions, dóna lloc un augment significatiu de l'activació plaquetària. S'inhibeix mitjançant la formació de AMPc, a través de la unió al receptor α_2-adrenèrgic de la plaqueta i a la proteïna GαZ. Una disminució del nombre de receptors d'epinefrina cursa amb complicacions hemorràgiques moderades.



3) FASE D'ESTABILITZACIÓ:

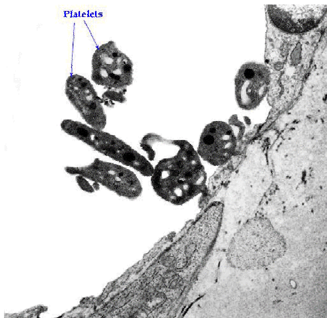
Es creen ponts entre les plaquetes adjacents de manera que les molècules alliberades per les plaquetes permetin la transferència d'informació a altres plaquetes. Els ponts entre les plaquetes es produeixen mitjançant la integrina GPIIb/IIIa que s'uneix al fibrinogen (agregació plaquetària). Les senyals transmeses en aquesta fase són bàsiques: es produeix una reorganització del citoesquelet, es forma i s'estabilitzen els agregats plaquetaris i a la membrana de la plaqueta es desenvolupa una activitat pro coagulant permetent la formació de la xarxa de fibrina i l'estabilització i retracció del tap plaquetari. La deficiència d'aquest receptor (GPIIb/IIIa) està associada a la malaltia de

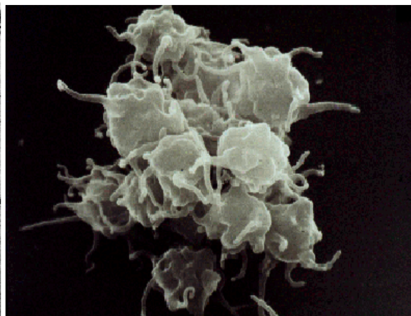
Glanzmann, que es caracteritza per una diàtesi hemorràgica que pot anar de moderada fins a molt severa.



1. Fase d'iniciació 2. Fase d'extensió 3. Fase d'estabilització

FvW Fg Gp Ib Gp IIbIIIa



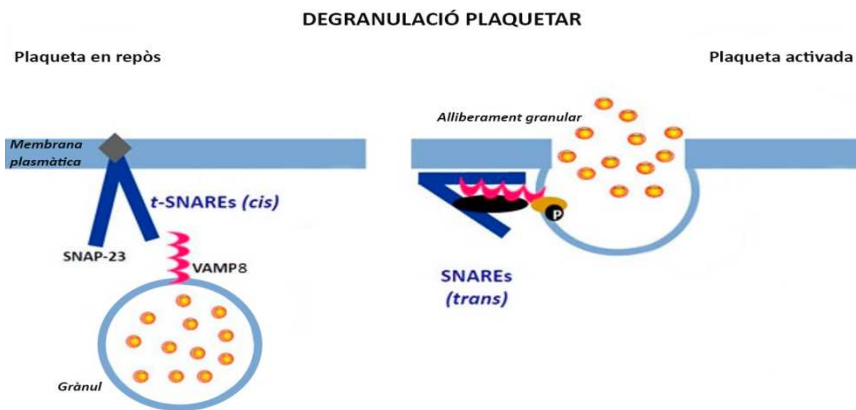


Adhesió plaquetària Agregació plaquetària

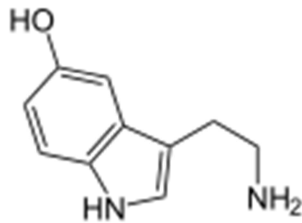
1.2.3 Proteïnes implicades en la secreció dels grànuls plaquetars:

L'agregació en resposta a l'epinefrina depèn de l'alliberament de la càrrega que contenen els grànuls plaquetars. Aquest alliberament es facilita per la interacció de proteïnes integrals de la membrana plasmàtica i dels grànuls, conegudes com t-SNARE (target-*Soluble NSF Attachment Protein Receptors*) i v-SNARE (vesicle-*Soluble NSF Attachment Protein Receptors*) respectivament. Les v-SNARE i les t-SNARE formen un complex heteromeric que s'estén sobre les dues bicapes i provoquen la fusió de les membranes permetent l'alliberament del contingut granular [2].

Human vesicle-associated membrane protein 8 (**VAMP-8 o Endobrevina**) és una v-SNARE que es requereix per l'alliberament dels grànuls plaquetaris. Anàlisis de plaquetes de ratolins knockout per VAMP8 indiquen que aquesta v-SNARE és la principal SNARE involucrada en la secreció dels grànuls densos, grànuls α i lisosomes [3].



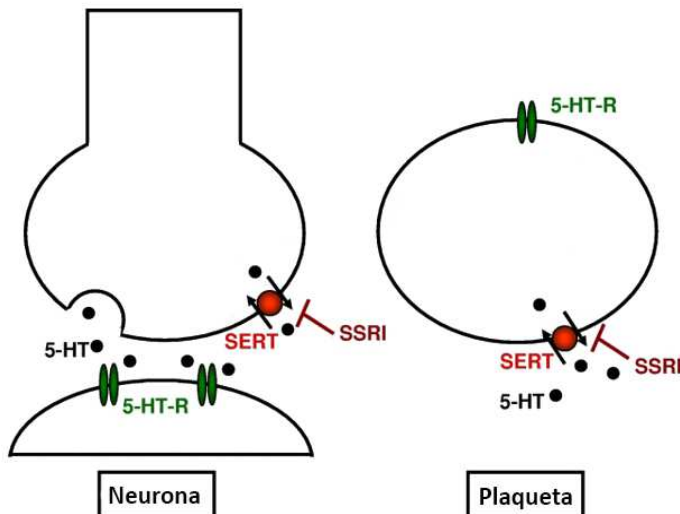
La **serotonina** (5-HT) és un neurotransmissor que se sintetitza en les neurones serotoninèrgiques i s'emmagatzema en els grànuls densos de les plaquetes. El seu alliberament dels grànuls densos és crític per l'activació plaquetària i per la vasoconstricció. S'ha demostrat que no és un agonista dèbil sinó que accentua l'activació plaquetària, potencia respostes procoagulants en sang humana i incrementa la trombogènesi de superfícies vasculars lesionades [4].



Serotonina (5-HT)

Un defecte del contingut dels grànuls densos causa el síndrome de Hermansky-Pudlack que cursa amb una diàtesi de sagnat de mitja a moderada. Es caracteritza també per una hipopigmentació de la pell i el cabell i una pèrdua de l'agudesa visual. Els portadors heterozigots d'aquest síndrome no tenen manifestacions clíniques.

El transportador de la Serotonina (**SERT**) és una proteïna de membrana que regula els nivells de Serotonina permetent el seu reciclatge. Es troba tant a la membrana de les neurones com a la membrana de les plaquetes. Els inhibidors de la recaptació de la serotonina (SSRI) actuen bloquejant SERT llavors disminueix el reciclatge de Serotonina [4].

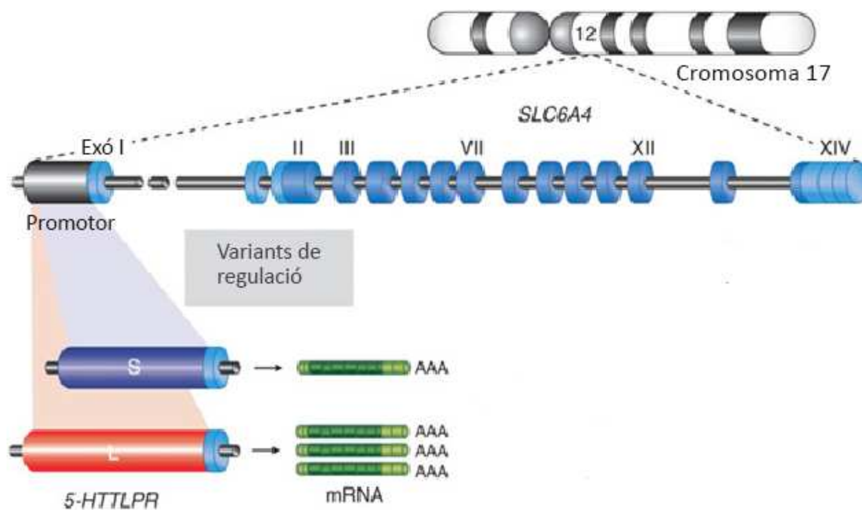


S'han descrit els polimorfismes *rs1010* del gen que codifica per VAMP8 i el *5-HTTLPR* de *SLC6A4* del transportador de la serotonina que estan relacionats amb els nivells d'aquestes proteïnes i el risc d'infart de miocardi.

El polimorfisme A → G (*rs 1010*) de VAMP8 està localitzat a la regió 3'UTR del gen de VAMP8 (al cromosoma 2). L'al·lel de risc d' infart de miocardi és el G i l'al·lel de no risc el A.

El polimorfisme de *SLC6A4* (SERT) està localitzat al promotor de *SLC6A4* (gen de SERT) (al cromosoma 17). És un polimorfisme de repetició de longitud (*5-HTTLPR*): la variació curta té 14 repeticions de la seqüència (S) i la llarga té 16 repeticions (L).

La variant SS s'associa a menor transcripció (nivells disminuïts de SERT) i LL s'associa a major transcripció (nivells alts de SERT).



1.3. L'ENDOTELI I L'HEMOSTÀSIA:

L'endoteli és un epiteli pla simple que forma la capa interna dels vasos sanguinis. En un humà de talla mitjana pesa aproximadament 1 kg, cobreix una superfície d' entre 4000 a 7000 m^2 i està composat d'1 a $6x10^{13}$ cèl·lules. Durant molts anys s'ha considerat com una membrana inert que regulava la permeabilitat de la paret del vas sanguini però actualment es considera un

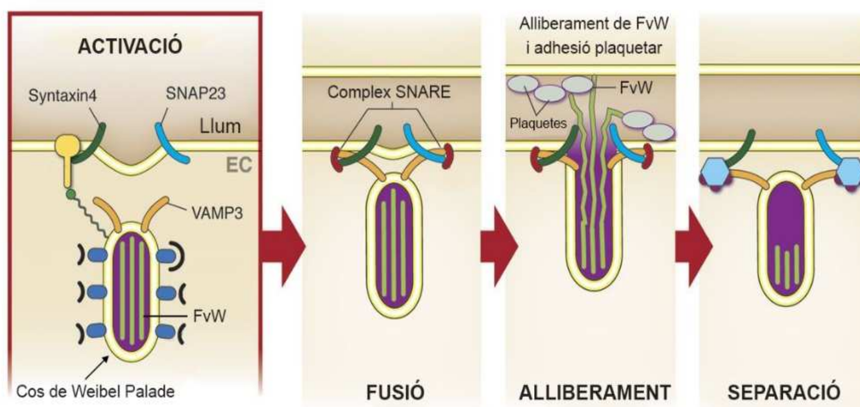
òrgan dinàmic, heterogeni i disseminat que té funcions secretores, sintètiques, metabòliques i immunològiques [5].

Així doncs l'endoteli participa en el control del trànsit cel·lular, la regulació del to vasomotor, l'hemostàsia i el creixement dels vasos sanguinis. Totes aquestes funcions es realitzen mitjançant receptors que es troben en la superfície de cada cèl·lula endotelial permetent la interacció cèl·lula-cèl·lula i cèl·lula-matriu, molts d'ells són presents també en les membranes cel·lulars de les plaquetes.

1.3.1 LA CÈL·LULA ENDOTELIAL:

Les cèl·lules endotelials són aplanades i tenen un nucli també aplanat. La part nuclear és més gruixuda i fa prominència a la llum. La part perifèrica és molt prima, les membranes que donen a la llum o als teixits estan separades per un gruix de citoplasma de 0,2 a 0,4 µm. Tenen aparell de Golgi, algunes mitocòndries i reticle endoplasmàtic, però l'orgànul característic són els cossos de Weibel Palade (on s'emmagatzema i s'allibera el FvW).

El FvW s'allibera mitjançant exocitosi dels cossos de Weibel Palade. Aquesta exocitosi es facilitada per l'acció d' SNAREs, en concret la t-SNARE Syntaxin 4 i la v-SNARE VAMP3. El FvW alliberat permetrà l'adhesió plaquetària [6].



Activació endotelial: es defineix com la capacitat de la cèl·lula endotelial d'adquirir noves funcions sense dany o divisió cel·lular. Les cèl·lules endotelials

no activades expressen: [5] activitat anticoagulant, anti-adhesiva i vasodilatadora. En canvi les cèl·lules endotelials activades expressen: activitat procoagulant, pro adhesiva i vasoconstrictora. Així doncs les cèl·lules endotelials tenen diverses funcions:

1.- Regulació del to vascular: mitjançant la secreció de substàncies tant vasodilatadores con vasoconstrictores.

a) Òxid nítric (NO): és un vasodilatador. La seva activitat està incrementada per la hipòxia i la trombina. Inhibeix l'adhesió de leucòcits i plaquetes sobre l'endoteli, d'aquesta manera s'inhibeix l'agregació plaquetària. Les catecolamines, vasopressina, bradicinina, histamina, serotonina i trombina estimulen el seu alliberament.

b) Prostaciclines ($PGI_{2)}$: és un metabòlit de l'àcid araquidònic. Regula la vasoconstricció induint la relaxació muscular. No se sintetitza al endoteli sinó que s'allibera on hi ha lesió vascular en resposta a la hipòxia o al *shear stress*. Té un efecte sinèrgic amb el NO. També inhibeix l'agregació plaquetària essent un dels antiagregants més potents.

c) Endotelines: Són pèptids de 21 aminoàcids. El flux sanguini és el regulador més potent de la producció i alliberament de les endotelines. Quan augmenta el flux sanguini es produeix una vasodilatació: augmenta la producció i alliberament de NO i disminueix la síntesi i alliberament d'endotelines per part de la cèl·lula endotelial.

2.- Control del trànsit cel·lular: mitjançant les propietats pro i anti adhesives de l'endoteli.

Degut a un alliberament constant de NO els elements que circulen per la sang no s'adhereixen a l'endoteli, l'endoteli sa és doncs antiadherent. En presència de citoquines pro inflamatòries s'exposen receptors (molècules d'adhesió) que mediaran les interaccions cèl·lula endotelial-cèl·lula endotelial, cèl·lula endotelial-matriu, plaquetes-cèl·lula endotelial i leucòcits-cèl·lula endotelial.

Hi ha diverses molècules d'adhesió cel·lular que són importants per la embriogènesi, el creixement i la diferenciació cel·lular i la inflamació (la participació de totes elles permet la migració dels leucòcits al focus inflamatori).

a) Immunoglobulines: participen en la migració dels leucòcits als teixits. Trobem ICAM-1 i ICAM-2 que són molècules d'adhesió intercel·lular, VCAM que són molècules d'adhesió de cèl·lules vasculars i PECAM que són molècules d'adhesió plaqueta-cèl·lula endotelial.

b) Integrines: Participen en la interacció cèl·lula-cèl·lula i cèl·lula-matriu. Per exemple: la glicoproteïna IIbIIIa (receptor de fibrinogen), $\alpha_v\beta_3$ (receptor de la vitronectina), $\alpha_5\beta_1$ (receptor de la fibronectina) i GP IaIIa (receptor del col·lagen).

c) Selectines: són molècules d'adhesió que s'exposen a les superfícies cel·lulars, s'anomenen depenent de la cèl·lula en la que estan presents, així doncs trobem: E-Selectines (selectina endotelial), P-selectina (selectina plaquetària) i L-Selectina (selectina leucocitària). S'activen per citoquines inflamatòries.

d) Caderines: promouen l'adhesió cèl·lula-cèl·lula mitjançant el Ca^{2+}.

3.- Paper de la cèl·lula endotelial en l'hemostàsia:

1.- En l'inici de la coagulació: estudis in vitro demostren que les citoquines, la trombina i la hipòxia indueixen l'expressió de FT en les cèl·lules endotelials. Per altra banda, el factor von Willebrand (FvW) és sintetitzat tant pels megacariòcits com per la cèl·lula endotelial.

2.- En la regulació de la generació de trombina mitjançant:

a) inhibidors de serinoproteases : antitrombina III (ATIII) i cofactor II de l'heparina (HCII). La matriu que envolta l'endoteli conté heparan sulfat (anàleg fisiològic de la heparina) i glicosaminoglicans. Ambdós promouen l'acció de l'ATIII per a inhibir la trombina.

b) Sistema de la proteïna C. Les cèl·lules endotelials expressen trombomodulina (TM). La TM forma un complex amb la trombina que és altament anticoagulant.

c) Inhibició de la via del FT. Les cèl·lules endotelials sintetitzen l'inhibidor de factor tissular (TFPI). El TFPI s'allibera de les cèl·lules endotelials per acció de l'heparina

3.- En la fibrinòlisi: les cèl·lules endotelials sintetitzen activadors i inhibidors del sistema fibrinolític:

a) el t-PA (activador tissular del plasminogen): la trombina, l'oclusió venosa i la vasopressina augmenten la seva síntesi.

b) el PAI (inhibidor de l'activador del plasminogen): La síntesi de PAI és estimulada per la trombina, l'endotoxina i diverses citoquines. La cèl·lula endotelial en repòs no expressa pràcticament inhibidor, així doncs el fetge seria la major font pel PAI plasmàtic.

c) el uPAR (receptors per l'activador del plasminogen tipus uroquinasa).

La resposta inicial de l'endoteli a l'endotoxina augmenta els nivells de t-PA que són ràpidament inhibits pel PAI. Treballs en cèl·lules endotelials en cultiu suggereixen que l'endoteli intacte és pro fibrinolític per tant contribueix al manteniment de la sang fluida.

Podem definir la **disfunció endotelial** com canvis fisiopatològics en l'estructura i funció de les cèl·lules endotelials que ocasionen la pèrdua d'equilibri entre:

a) Anti trombòtic ⟶ pro trombòtic
b) Vasorelaxació ⟶ vasoconstricció
c) Inhibició i estimulació dels factors de creixement
d) Antiinflamatoris i pro inflamatoris

La manifestació clínica inicial de la disfunció endotelial és la vasoconstricció, la formació del coàgul i la hipertensió. A llarg termini es produeix l'aterosclerosi.

L'endoteli està permanentment responent a diferents alteracions a nivell extracel·lular, no hauríem de parlar doncs d'endoteli activat o no activat, sinó d'un espectre d'activació continu que succeiria tant en condicions fisiològiques com en resposta a diversos estadis de malaltia.

1.3.2 Interacció plaquetes-endoteli-proteïnes plasmàtiques [1]**:**

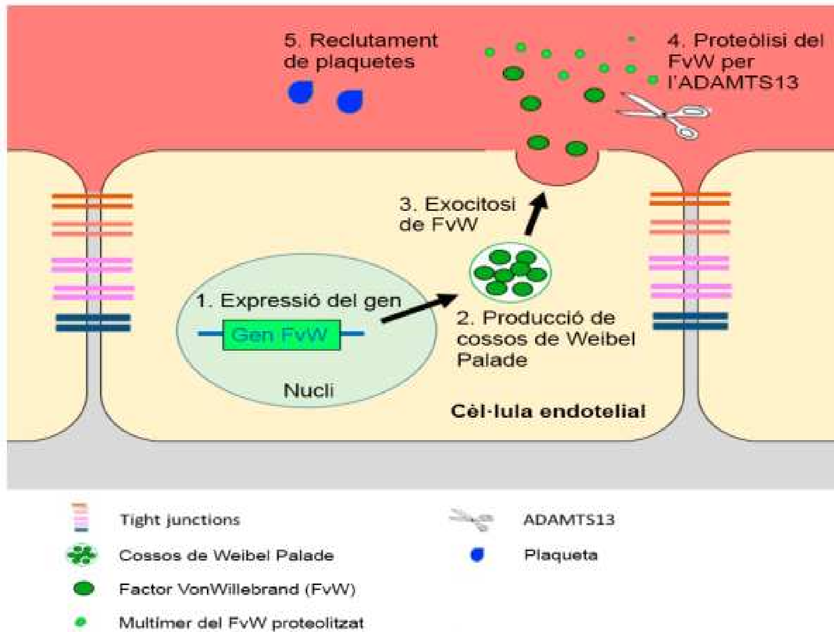
Factor von Willebrand

El FvW és una proteïna multimèrica d'elevat pes molecular. Se sintetitza tant a l'endoteli com als megacariòcits, i es transporta per les plaquetes. Hi ha estudis que demostren que la majoria de FvW plasmàtic prové de la síntesi endotelial. L'estrés, l'exercici, les hormones, etc provoquen que les plaquetes alliberin el FvW a la sang on s'uneix al factor VIII. La seva activitat contribueix al fenomen de l'adhesió plaquetària, actuant com a pont entre la plaqueta i el subendoteli com ja s'ha explicat anteriorment. És doncs un marcador de disfunció endotelial i activació plaquetària.

ADAMTS13

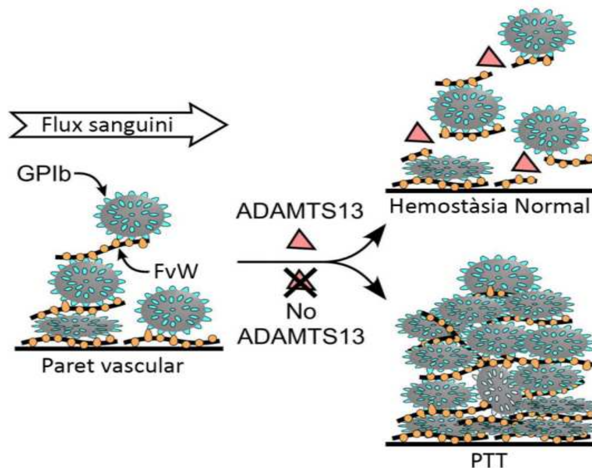
L'ADAMTS13 (*a disintegrin and metalloproteinase with thrombospodin-1-like domains*) és una metalloproteasa que regula els nivells de FvW produint una proteòlisi parcial dels multimers d'alt pes molecular que són els necessaris per a l'adhesió plaquetària. Degrada aquest multimer en formes més petites i menys actives trencant l'enllaç peptídic Tyr1605-Met1606 del FvW.

L'ADAMTS13 se sintetitza al fetge i a les cèl·lules endotelials, s'allibera a la circulació general en forma activa, la seva concentració plasmàtica és aproximadament de 1μg/mL i la seva vida mitja és de 2-3 dies.



En condicions normals, el FvW i l'ADAMTS13 circulen pel plasma i es probable que la inactivació dels multimers d'alt pes molecular sigui molt petita. Aquesta inactivació és 1000 vegades més ràpida i eficient quan el FvW s'expressa en l'endoteli vascular lesionat. Hi ha un equilibri *in vivo* constant entre la quantitat de multimers d'alt pes molecular de FvW i l'alliberament d'ADAMTS13 per les cèl·lules endotelials. D'aquesta manera, l'ADAMTS13 prevé la formació de coàguls en l'endoteli activat tant de capil·lars venosos com d'arterioles.

L'ADAMTS13 redueix l'adhesió plaquetària i l'agregació, en estudis recents s'han demostrat també un efecte antiinflamatori. Nivells baixos d'ADAMTS13 (ja sigui per deficiència o per presència d'inhibidor) provoquen una circulació de multimers ultra llargs, promovent llavors l'agregació plaquetària i l'aparició de microtrombes (síndrome de la púrpura trombòtica trombocitopènica (PTT)).



1.4 CÈL·LULES IMPLICADES EN LA PROPAGACIÓ DE LA TROMBOSI VENOSA EN RATOLINS [7]

Estudis recents en ratolins [7] demostren que els monòcits, neutròfils i les plaquetes cooperen en la iniciació i la propagació de la trombosi venosa *in vivo*. Es demostra per primer cop que les plaquetes i el FvW són essencials per a la formació del coàgul venós experimental.

En aquest treball s'usa un nou model de restricció del flux induint trombosi venosa en ratolins després d'una estasis venosa. Després de 2 hores de restricció del flux es visualitza mitjançant microscòpia intravital que les plaquetes s'adhereixen i després de 6 hores les plaquetes s'adhereixen directament a l'endoteli intacte o als leucòcits (mitjançant el receptor GPIb). Les plaquetes i els leucòcits formen petits agregats en contraposició a la trombosi arterial on les plaquetes s'acumulen en pocs minuts formant agregats grans.

Es fa palès que la reducció del flux sanguini indueix un fenotip pro inflamatori endotelial on s'inicia el reclutament de cèl·lules immunes, particularment de neutròfils i monòcits. Aquestes cèl·lules immunes inicien la formació del coàgul per a desenvolupar trombosi venosa mitjançant dos mecanismes que requereixen la participació de a) el FvW i b) les plaquetes:

a) Els leucòcits reclosos comencen la formació de fibrina mitjançant l'expressió del FT dels monòcits. Aquest fenotip pro infamatori activa les cèl·lules

endotelials mitjançant l'exposició de molècules d'adhesió com el FvW i la P-selectina endotelial.

b) L'alliberament de trampes extracel·lulars de neutròfils (NETs). Les NETs són xarxes de cromatina que s'alliberen del nucli dels neutròfils i que retenen tant cèl·lules sanguínies i bacteris com proteïnes (per ex FvW, FXII) donant lloc a un estat pro trombòtic. Les plaquetes interaccionen amb les leucòcits mitjançant la GPIb, són critiques per la propagació de la trombosi venosa doncs permeten l'acumulació de leucòcits i promouen la formació de NETs, les quals desencadenen la propagació del coàgul mitjançant el FXIIa.

Així doncs podem concloure que la interacció de les plaquetes amb els leucòcits mitjançant la GPIb exerceix una funció dual durant el desenvolupament de la trombosi venosa doncs per una banda facilita el reclutament dels leucòcits i per altra estimula la formació de NETs dels neutròfils.

1.5 EL GRUP SANGUINI ABO I EL FvW:

Aproximadament el 30% de la variabilitat genètica que influeix en els nivells de FvW en el plasma està influenciat pel grup sanguini ABO. Molts treballs descriuen la influència del grup ABO en els nivells de FvW i FVIII plasmàtic [8-11]. El nostre grup va trobar que el grup ABO i els nivells de FVIII plasmàtics eren factors de risc independents de trombosi venosa [12]. Els individus de grup sanguini O tenen un 25 % més baix els nivells de FvW que els de grup sanguini no-O [13-15]. S'han suggerit diverses hipòtesi per explicar-ho: el grup sanguini ABO podria afectar a la ràtio de síntesi/secreció de FvW. Els individus de grup O podrien tenir augmentat el seu aclariment del plasma i una supervivència menor de la proteïna.

Els antígens del grup sanguini ABO (determinants A, B i H) són molècules complexes de carbohidrats [16]. Els al·lels *A* i *B* codifiquen activitats glicosiltransferases A i B les quals converteixen el antigen precursor H en determinants A o B. L'al·lel *A* afegeix una N-acetilgalactosa i l'al·lel *B* afegeix una D-galactosa al antigen H. Els individus de grup sanguini O no tenen les enzims transferases i per tant continuen expressant la molècula H. Una deleció en el gen *A* es expressat com al·lel A_2. També, com a resultat d'una mutació *frameshift* la glicosiltransferasa codificada pel al·lel A_2 té un domini

carboxiterminal addicional que sembla ser el responsable per la reducció dràstica en l'activitat enzimàtica [17].

CAPÍTOL 2

HIPÒTESI i OBJECTIUS

CAPÍTOL 2

2.1 HIPÒTESI:

La patologia associada a l'hemostàsia primària ha estat clàssicament relacionada amb diàtesi hemorràgica (malaltia de von Willebrand, Síndrome de Bernard-Soulier, malaltia de Glanzmann, trombopènia, etc) on s'ha valorat el paper de les plaquetes i del factor von Willebrand en una defectuosa agregació i/o adhesió plaquetària. També ha estat àmpliament estudiat el paper de les plaquetes en la trombosi arterial.

Ja que el paper de les plaquetes és fonamental en l'inici i la formació del coàgul és possible que tinguin un paper important en la patogènia de la trombosi venosa. Treballs experimentals en ratolins demostren que les plaquetes i el FvW juguen un paper molt important en l'inici i el desenvolupament de la trombosi venosa [7]. En aquesta tesi es vol investigar sobre el paper d'aquests paràmetres en patologia trombòtica venosa humana.

Recentment s'ha introduït el concepte d'hiperagregabilitat plaquetària on trobem individus amb plaquetes que agreguen a dosis molt petites d'inductor (ADP o/i Epinefrina). La agregabilitat a dosis baixes d'epinefrina (EPI) i/o d'ADP en la població normal dona lloc a dues poblacions diferenciades: individus amb hiperreactivitat plaquetària (agregació >60%) i individus amb hiporreactivitat plaquetària (agregació <40%) [18,19].

La reactivitat plaquetària (hipo i hiperreactivitat plaquetària) es pot mesurar a) mitjançant l'agregació plaquetària activada per agonistes en plasma ric en plaquetes (PRP) com s'ha indicat anteriorment o b) mitjançant test que usen sang total. Aquests test en sang total poden ser o bé agregacions en sang total (usem l'analitzador Multiplate®) o bé mitjançant test basats en l'adhesió plaquetària (usem l'analitzador PFA-100®). El PFA-100® és un analitzador que mesura el funcionalisme plaquetari. Es mesura en segons (temps d'oclusió) i aquest temps d'oclusió és inversament proporcional a la capacitat funcional de les plaquetes. El nostre grup ha descrit que temps d'oclusió curts de l'analitzador PFA-100® s'associen a risc trombòtic venós [20] suggerint que els

temps d'oclusió mesurats en el PFA-100® podrien ser un marcador d'adhesió plaquetària.
La hiperreactivitat plaquetària s'ha trobat associada a trombosis arterial i venosa i està relacionada amb els nivells de Serotonina, del seu transportador (SERT) i de VAMP 8 [19,21].

Aquesta hiperreactivitat sembla ser un fenomen global, on individus amb un increment d'agregació plaquetar en resposta a l' EPI tenen probablement també una resposta elevada a altres agonistes plaquetaris. S'ha descrit que una coestimulació de serotonina i una concentració submàxima d' EPI augmenta l'agregació en una proporció més alta que quan l'indueixes només amb EPI mantenint-se en la població la resposta bimodal [19]. El nombre d'hiperreactius quan s'utilitzen els dos inductors augmenta de manera significativa. Quan uses serotonina com a agonista sol, els individus tenen una resposta modesta, recolzant el fenomen que les plaquetes son reactives a la serotonina nomes si són activades per altres agonistes com exemple EPI. El mateix succeeix quan afegeixes serotonina a l'ADP, s'augmenta també l'hiperrectivitat plaquetària i s'ha observat que es produeix un efecte general pro coagulant [4].

Altres estudis defineixen també la hiperreactivitat plaquetària com un fenomen global [22]. Descriuen que individus sans amb hiperreactivitat plaquetària a l' EPI tenen una funció plaquetària augmentada tant a nivell d'adhesió (s'incrementa l'aglutinació en resposta a baixes dosis de ristocetina) com d'activació (s'incrementa l'expressió de la P-selectina després de l'alliberament granular) i d'agregació (s'incrementa l'agregació en resposta a múltiples estímuls diferents).

En individus hiperreactius s'ha observat nivells més elevats de SERT i un augment en l'afinitat d'unió de serotonina-SERT [19]. També en individus amb hiperreactivitat plaquetària els nivells de VAMP 8 estan augmentats [21].

Els polimorfismes *rs1010* de VAMP8 i el *5-HTTLPR* de *SLC6A4* del transportador de la serotonina estan relacionats amb els nivells d'aquestes proteïnes, amb la hiperreactivitat plaquetària i amb el risc d'infart de miocardi [23-25].

A més a més diversos estudis han demostrat que la disminució d'ADAMTS13 i l'augment del factor von Willebrand són factors de risc de trombosi arterial [26-

30], però hi ha poca evidència d'aquesta relació i la trombosi venosa mostrant altrament resultats controvertits [31]. Es valora també la relació entre el grup sanguini ABO i els nivells d'aquestes proteïnes.

Per altra banda, ja que el nostre grup ha descrit que temps d'oclusió curts de l'analitzador PFA-100® s'associen a risc trombòtic venós [20] es valora també la relació entre el grup sanguini ABO i aquest fenotip.

Per tots aquests motius esmentats anteriorment creiem que es plausible plantejar la hipòtesi de la implicació de l'hemostàsia primària en l'etiopatogènia de la trombosi venosa.

2.2 OBJECTIUS:

L'objectiu fonamental és valorar el possible paper pro trombòtic de les plaquetes i de les proteïnes plasmàtiques implicades en l'hemostàsia primària en la trombosi venosa. Per això hem estudiat en distintes poblacions amb trombosi venosa, paràmetres de l'hemostàsia primària no estudiats prèviament.

Concretament:

a) El fenomen d'hiperagregabilitat plaquetària i la trombosi venosa.
b) Els nivells de VAMP8, SERT i SEROTONINA i la seva relació amb el funcionalisme plaquetari i la trombosi venosa.
c) Els polimorfismes *rs1010* de VAMP8 i el *5-HTTLPR* de *SLC6A4* del transportador de la serotonina i la trombosi venosa.
d) Els nivells de factor von Willebrand i ADAMTS13 i la trombosi venosa.
e) El grup sanguini ABO en relació al funcionalisme plaquetari, el FvW i l'ADAMTS13.
f) Valorar si tots aquests paràmetres poden ser possibles marcadors de risc trombòtic.

CAPÍTOL 3

MATERIALS I MÈTODES

3.1 POBLACIONS ESTUDIADES

3.1.1 Població RETROVE (Capítol 4.1 ,4.2 i annex capítol 4.2 i 4.3):

Projecte RETROVE (Risc de "Enfermedad TROmboembólica VEnosa"): Es tracta d'un estudi cas-control, aleatoritzat i prospectiu amb pacients amb malaltia tromboembòlica venosa. L'objectiu de l'estudi és definir un algoritme de risc de trombosi, en el qual s'inclouen dades clíniques, plasmàtiques, genètiques i epigenètiques. Aquest algoritme ha de permetre una quantificació individual del risc de malaltia tromboembòlica venosa, mitjançant un *score* de trombogenicitat. S'ha reclutat, durant el període de 2012 a 2016, una mostra de 400 controls sans (majors de 18 anys) amb una mitjana d'edat de 49±18 anys (194 homes i 206 dones) i 400 pacients amb trombosi (majors de 18 anys i sense límit superior d'edat) amb una mitjana d'edat de 64±18 anys (196 homes i 204 dones). Els episodis tromboembòlics observats són tant espontanis com secundaris a situacions de risc conegudes. Tots ells han estat diagnosticats per mètodes objectius. S'han exclòs els casos associats a càncer actiu (trombosis paraneoplàsiques) o a hepatopatia crònica (pel seu efecte en els paràmetres plasmàtics de l'hemostàsia).

Els controls són individus sense antecedents personals de malaltia tromboembòlica venosa ni arterial (IAM, ictus, malaltia arterial perifèrica), sense antecedents de càncer ni hepatopatia crònica. Del mateix origen geogràfic i ètnic (caucàsics) que els casos. La seva distribució d'edat i sexe s'ha ajustat a la piràmide poblacional espanyola (cens 2001) de majors de 18 anys. Hi ha, intencionadament, un no aparellament en edat i sexe entre els casos i els controls per a poder avaluar la importància d'aquestes variables en el risc de trombosi i la seva eventual inclusió en el algoritme de risc individual. Cap dels individus havia rebut aspirina ni AINES en els 10 dies anteriors a l'extracció sanguínia.

3.1.2 Població cas-control d'individus amb trombosi venosa (Capítol 4.3):

L'estudi està compost per 250 pacients i 250 controls. Els pacients s'han inclòs consecutivament i han hagut de patir la seva primera trombosi venosa abans

dels 70 anys. S'han exclòs els pacients amb càncer o hepatopatia crònica o síndrome nefròtic. Els controls s'han inclòs seguint els següents criteris: edat similar (±10 anys), mateix sexe, no parentiu genètic i no història personal ni familiar de trombosi venosa. Els controls són de la mateixa àrea geogràfica que els pacients (tots amb cognoms d'origen espanyol). El reclutament es va realitzar entre els anys 1997-2002. Entre els 250 pacients 113 son homes i 137 dones. La mitjana d'edat és de 47,6±14 anys. En quan als controls 109 són homes i 141 dones. La seva mitjana d'edat és de 49,6±14,9 anys.

3.1.3 Població GAIT 2 (Capítol 4.4):

El projecte GAIT-2 (Genetic Analysis of Idiopathic Thrombophilia-2) inclou 35 famílies grans espanyoles seleccionades (durant 2006-2009) a partir d'un propositus amb trombofilia venosa idiopàtica. La trombofilia es va definir com a: múltiples episodis trombòtics venosos (almenys un espontani), un episodi espontani de trombosi venosa amb un parent de primer grau també afectat, o trombosi venosa abans dels 45 anys. Es va considerar el propositus de trombofilia venosa idiopàtic quan es van excloure totes les causes biològiques conegudes de trombosi (durant el període de reclutament): deficiència de ATIII, de Proteïna S i C, resistència a la Proteïna C activada, Factor V Leiden, mutació del polimorfisme PT20210A, disfibrinogenemia, anticoagulant lúpic, i anticossos antifosfolípids. Aquests factors trombofílics també eren absents en tots els parents afectats.

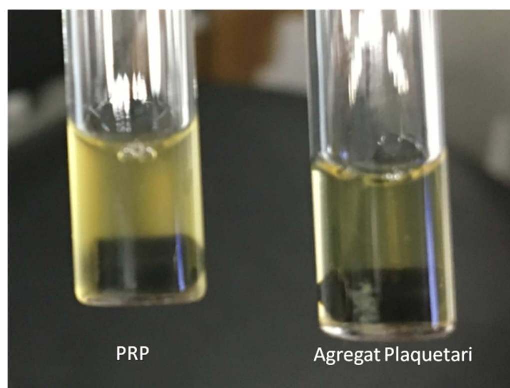
Per tal de maximitzar la detecció dels defectes genètics, les famílies incloses havien de tenir com a mínim 10 individus en tres o més generacions. Els membres de la família van ser entrevistats per un metge per a confirmar que no havien pres fàrmacs antiplaquetaris en les últimes dos setmanes o fàrmacs amb efecte sobre la funció plaquetar com AINES o SSRI (serotonin reuptake inhibitor) en la última setmana. Dels individus estudiats 465 eren homes i 470 eren dones. La mitjana d'edat era de 39,5 anys (mínim 2,6, màxim 101, SD 21,4) i 197 d'ells tenien 18 o menys anys. Hi havia 86 individus amb trombosi venosa, 47 amb trombosi arterial, i 13 amb ambdós tipus de trombosi (venosa i arterial).

3.2 TÈCNIQUES:

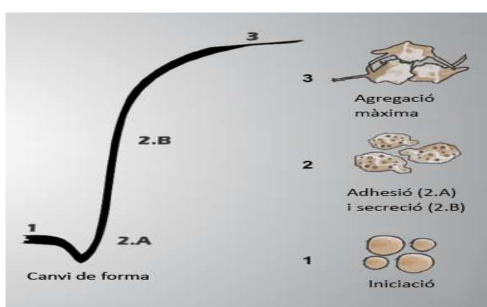
3.2.1 Agregacions en plasma ric en plaquetes (PRP): Mètode de Born

S'usa un agregòmetre de transmissió de llum (Light Transmission Aggregometry (LTA)):

LTA mesura l'agregació plaquetària en funció del grau de transmissió de la llum a través d'una suspensió de cèl·lules (les plaquetes). A mesura que les plaquetes comencen a agregar en resposta a un agonista plaquetari, la terbolesa de la suspensió es redueix i això fa que hi hagi un augment de la transmissió de la llum.



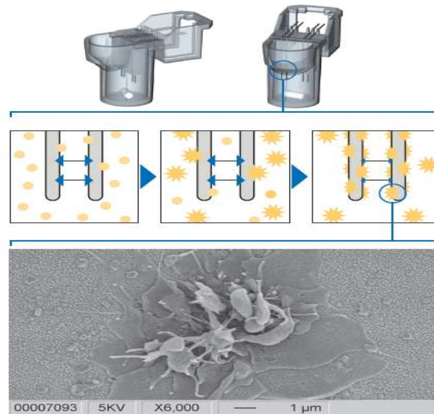
L'agregació plaquetària s'indueix per diferents agonistes, en el nostre cas ADP i EPI.
Proporciona informació de les diferents fases de l'activació plaquetària, des del canvi de morfologia fins a l'agregació, per això es segueix considerant el "gold standard".



Té l'inconvenient que les plaquetes estan aïllades de la resta de la sang total, estan en condicions de cisallament baixes i només agreguen després d'afegir l'agonista. Per tant no són condicions fisiològiques reals.

3.2.2 Agregacions en Sang total:

S'usa l'analitzador Multiplate® que mesura canvis en la impedància. S'afegeix sang total anticoagulada amb hirudina a les cubetes. La senyal de reacció en l'analitzador Multiplate® es desencadena per l'adhesió de les plaquetes activades a la superfície dels elèctrodes del sensor. Intenta reflectir la funció plaquetària "in vivo" que es produeix sobre les superfícies.

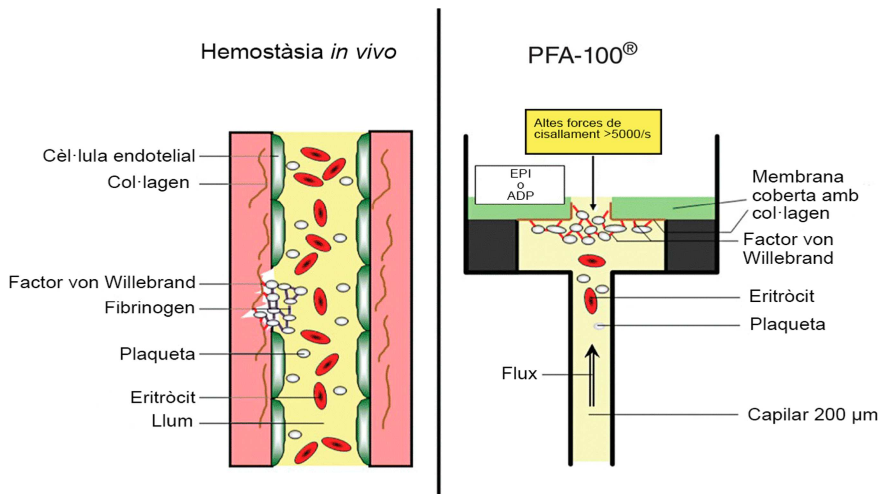


És més fisiològic que LTA però no dóna informació sobre les distintes fases de l'agregació. Té més interferències depenent de la "qualitat" de la sang (hemolitzada, lipèmica, etc).

3.2.3 Funcionalisme plaquetari:

S'usa l'analitzador PFA-100®. El PFA-100® simula la paret d'un vas sota condicions d'estrés fisiològiques. El dispositiu conté una membrana coberta amb col·lagen i també amb ADP (PFA_ADP) o amb epinefrina (PFA_EPI). Aquesta membrana té un forat a través del qual passa la sang anticoagulada. La presència d'aquests agonistes i les altes forces de cisallament fan que es produeixi l'adhesió, l'activació, l'agregació plaquetària, i finalment es formi un tap plaquetari estable que tapa el forat. El temps requerit per tapar el forat, en

segons, es el que es mesura (temps d'oclusió) i és inversament proporcional a la capacitat funcional de les plaquetes.



Detecta defectes plaquetars importants. Està influenciat pels nivells de FvW, pel grup sanguini ABO, per l'hematòcrit i el número de plaquetes.

3.2.4 VAMP8, SERT i serotonina:

Els nivells de **VAMP8** es van determinar amb el kit comercial: *Human Vesicle-associated membrane protein 8 (VAMP8) ELISA kit* de CUSABIO.

Les plaquetes es van aïllar prèviament: es va rentar el PRP amb tampó fosfat i posteriorment es va centrifugar a 4500 x g 10 min. El pellet plaquetari es va congelar a -40ºC. Abans de la determinació, el pellet plaquetari es va resuspendre amb Triton X-100 i es va refredar amb gel durant 1 hora. La concentració final de VAMP8 es va expressar en relació al número de plaquetes (pg/10^9 PLT).

Els nivells de **SERT** es van determinar amb el kit comercial: *Enzyme-linked Immunosorbent assay kit* per a *Serotonin Transporter (SERT)* de Uscn. Les plaquetes es van aïllar com s'ha descrit prèviament. La concentració final de SERT es va expressar també en relació al número de plaquetes (pg/10^9 PLT).

Els nivells de **SEROTONINA** es van determinar amb el kit comercial: *Serotonin ELISA kit* de IBL international. Les plaquetes es van aïllar com s'ha descrit prèviament però el pellet plaquetari es va resuspendre amb aigua destil·lada. De la mateixa manera la concentració final de serotonina també es va expressar en relació al número de plaquetes (ng/10^9 PLT).

3.2.5 ADAMTS13 i FvW:

Els nivells de **ADAMTS 13** antigènics es van mesurar amb el kit comercial TECHNOZYM ADAMTS 13 Antigen ELISA.

Els nivells de **FvW** antigènic es van mesurar amb el kit comercial Von Willebrand Factor Antigen test REAADS.

3.2.6 PFA-100® i la seva relació amb el locus *ABO*:

Pel fenotip del **PFA-100®** i la seva relació amb el **locus *ABO*** es va realitzar un estudi d'associació del genoma complet (GWAS) amb ≈ 10M de polimorfismes de nucleòtid únic (SNPs) amb els fenotips PFA_ADP i PFA_EPI. Es van genotipar les mostres de 934 individus.

3.3 ANÀLISI ESTADÍSTIC:

En l'anàlisi de la hiperagregabilitat plaquetària es va usar el coeficient de correlació d' Spearman per a calcular els valors entre el PFA-100® i l'agregació en PRP (mesurats per % Agregació Màxima (MA) i % àrea sota la corba (AUC)). En l'agregació amb PRP mitjançant el mètode de LTA: els resultats es van expressar en diferències de mediana per que no seguien una distribució normal (test de kolmogorov). Es va calcular el percentil 50 del percentatge AUC per l' EPI i el percentil 75 per l'ADP en els controls per a discriminar els individus amb hipo- o hiperagregabilitat plaquetària. Les freqüències entre els individus amb hipo o hiperagregabilitat plaquetària es van comparar amb el test de $\chi 2$. Es va realitzar una regressió logística condicional per a estimar el risc de trombosi venosa amb una Odds Ratio (OR) crua i ajustada per edat i sexe amb un interval de confiança del 95%. Les diferències entre controls i pacients en agregacions amb PRP i agregometria d'impedància en sang total es van calcular mitjançant el test de Wilcoxon. Es va aplicar una ANOVA de dos factors per calcular les diferències del fibrinogen relatives a l'agregabilitat plaquetària (individus hipo- i hiper-) per al grup individu (controls i pacients). També es va realitzar una ANOVA de dos factors per a calcular les diferències de PFA-100® relatives a la hiperagregabilitat plaquetària per al grup individu.

Els valors de VAMP8, SERT i SEROTONINA es van expressar en mediana i desviació interquartil (percentils 25 i 75). La comparació dels nivells entre pacients i controls es va realitzar amb el test de Mann-Whitney. Es va seleccionar el P75 com a punt de tall ($\geq$1930 pg/10^9 PLT) dels nivells de VAMP8 i ($\geq$784 (pg/10^9 PLT) dels nivells de SERT. Per a la Serotonina es va seleccionar el percentil 25 ($\leq$115 (ng/10^9 PLT). Es van valorar les diferències de les freqüències entre pacients i controls amb el test $\chi 2$. Es va estimar la OR crua i ajustada per edat, sexe i els factors que han estat associats prèviament amb trombosi venosa en la nostra població: Factor V Leiden i nivells de Factor VIII (percentil 90) mitjançant una regressió logística pas a pas.

En l'anàlisi dels nivells d'ADAMTS13 es va realitzar el test de la t-Student per a calcular les diferències entre els grups i el test de $\chi 2$ per comparar les freqüències. Es va estimar també la OR crua i ajustada tal com s'ha descrit prèviament. Els nivells de FvW i ADAMTS13 va ser comparats amb el grup ABO mitjançant una ANOVA.

En tots els anàlisi es van considerar estadísticament significatius els valors $p<0.05$.

En el GWAS del fenotip PFA-100® es van usar dues fases per a l'anàlisi d'associació dels fenotips quantitatius amb els genotips imputats. El primer va ser un *sreening* ràpid de les variants amb Matrix eQTL usant un model lineal. El GWAS de cada fenotip es va calcular ajustant-lo per edat. El sexe, el tabac, i els contraceptius orals no tenen influència en el fenotip PFA-100®, així doncs, no es van incloure com a covariables en l'estudi. Les variants amb una $p< 10^{-3}$ es van recalcular en una segona fase amb SOLARIUS i SOLAR-Eclipse usant mètodes de components de la variança. Només es van tenir en compte les p-valors calculades amb SOLAR doncs és considerat el *gold-standard* per a mostres amb arbre genealògics extensos. Per tal d'avaluar la significació estadística dels paràmetres del model de la variança, SOLAR usa l'enfocament de la màxima versemblança mitjançant el test de ràtio de versemblances.

CAPÍTOL 4

RESULTATS

INFORME DELS DIRECTORS DE LA TESI DE LA PARTICIPACIÓ DE LA DOCTORAND:

1er ARTICLE: PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK: RESULTS FROM THE RETROVE PROJECT.

Llobet, Dolors; Vallvé, Cristina; Tirado, Isabel; Vilalta, Noèlia; Carrasco, Marina; Oliver, Artur; Mateo, José; Fontcuberta, Jordi; Souto, Juan Carlos.

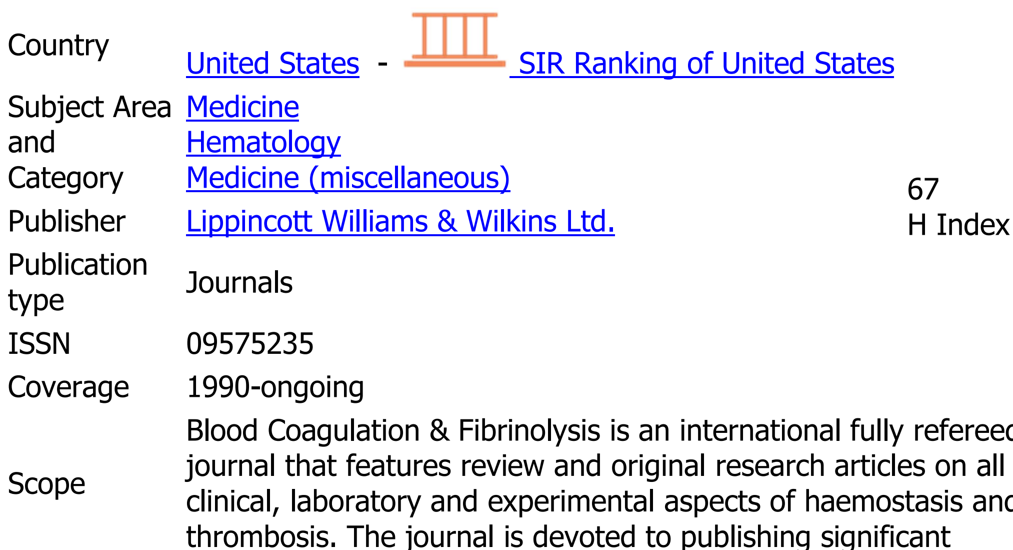
Sotmès al **Blood coagulation and Fibrinolysis**

Blood Coagulation & Fibrinolysis
Editorial Manager
Submissions Being Processed for Author Dolors Llobet, Ph D
Page: 1 of 1 (1 total submissions)

Action	Manuscript Number	Title	Initial Date Submitted	Status Date
Action Links		PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK: RESULTS FROM THE RETROVE PROJECT.	06/09/2020	06/09/2020

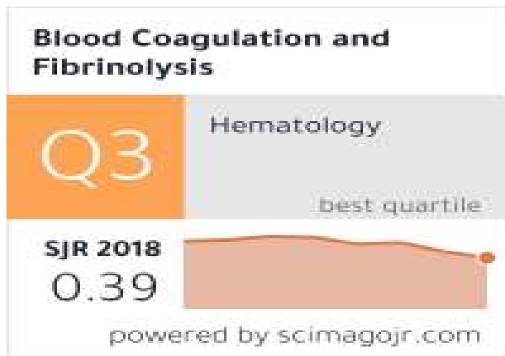
<< Author Main Menu

Descripció de les característiques de la revista **Blood Coagulation and Fibrinolysis**:



Country	United States - SIR Ranking of United States	
Subject Area and Category	Medicine Hematology Medicine (miscellaneous)	67 H Index
Publisher	Lippincott Williams & Wilkins Ltd.	
Publication type	Journals	
ISSN	09575235	
Coverage	1990-ongoing	
Scope	Blood Coagulation & Fibrinolysis is an international fully refereed journal that features review and original research articles on all clinical, laboratory and experimental aspects of haemostasis and thrombosis. The journal is devoted to publishing significant	

developments worldwide in the field of blood coagulation, fibrinolysis, thrombosis, platelets and the kininogen-kinin system, as well as dealing with those aspects of blood rheology relevant to haemostasis and the effects of drugs on haemostatic components.



2on ARTICLE: VAMP8 and SEROTONIN TRANSPORTER LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN A SPANISH FEMALE POPULATION. RESULTS FROM THE RETROVE PROJECT.

Dolors Llobet, Cristina Vallvé, Isabel Tirado, Noèlia Vilalta, Joaquin Murillo, Biel Cuevas, Lidia Roman, Marina Carrasco, Artur Oliver, Jose Mateo, Jordi Fontcuberta, Juan Carlos Souto.

Thrombosis Research 181 (2019) 99–105

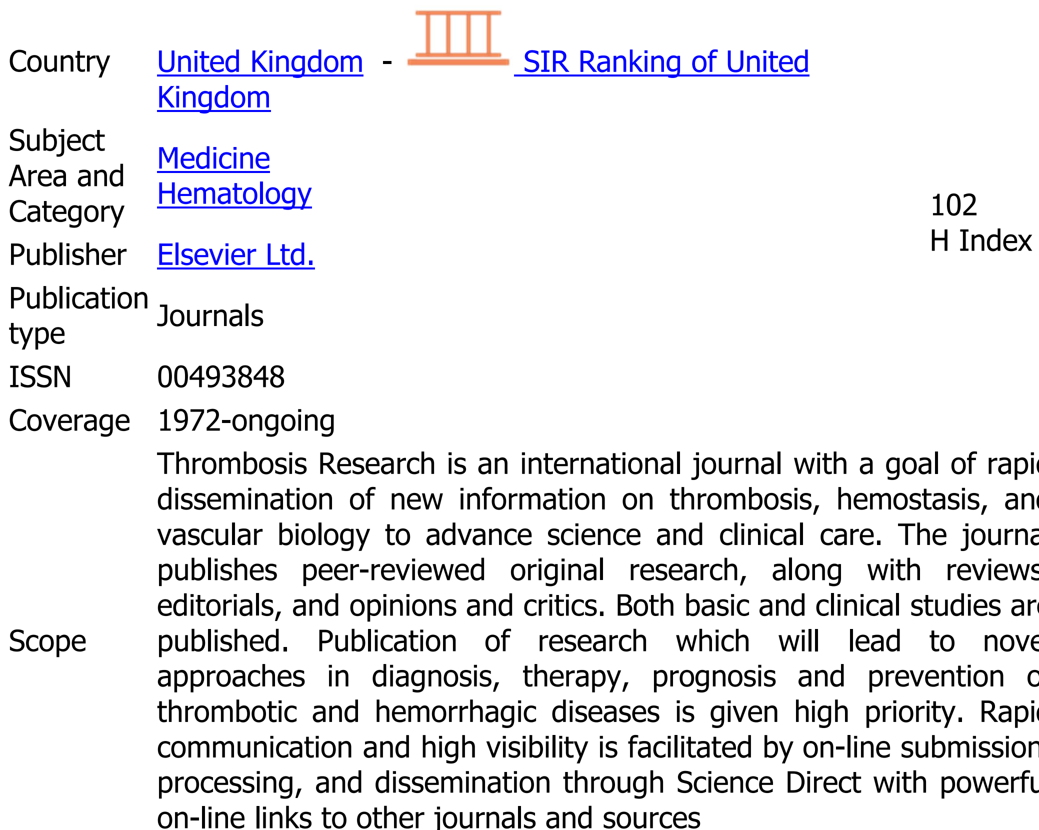
3 er ARTICLE: LOW ADAMTS13 LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN WOMEN.

D. Llobet*, I. Tirado, N. Vilalta, C. Vallvé, A. Oliver, M. Vázquez-Santiago, J. Mateo, J. Millón, J. Fontcuberta, J.C. Souto.

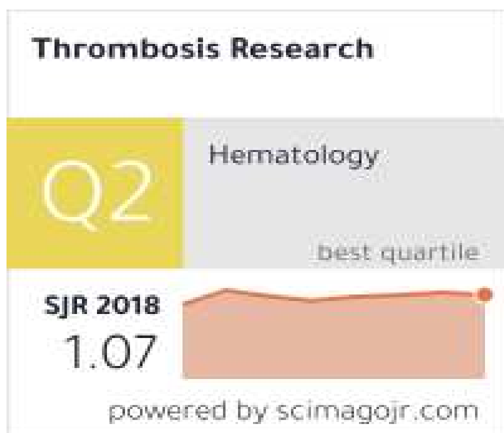
Thrombosis Research 157 (2017) 38–40

En els tres primers articles, la doctorand ha participat en el disseny del projecte. Ha realitzat la recerca i la recol·lecció de les mostres així com la realització de les tècniques de laboratori corresponents. Per altra banda també ha tingut accés a la base de dades des d'on ha realitzat i interpretat l'anàlisi estadístic. Per últim ha fet l'esborrany dels manuscrits i ha tingut un paper decisiu en la versió definitiva de les publicacions.

Descripció de les característiques de la revista **Thrombosis Research:**



Country	United Kingdom - SIR Ranking of United Kingdom	
Subject Area and Category	Medicine Hematology	102 H Index
Publisher	Elsevier Ltd.	
Publication type	Journals	
ISSN	00493848	
Coverage	1972-ongoing	
Scope	Thrombosis Research is an international journal with a goal of rapid dissemination of new information on thrombosis, hemostasis, and vascular biology to advance science and clinical care. The journal publishes peer-reviewed original research, along with reviews, editorials, and opinions and critics. Both basic and clinical studies are published. Publication of research which will lead to novel approaches in diagnosis, therapy, prognosis and prevention of thrombotic and hemorrhagic diseases is given high priority. Rapid communication and high visibility is facilitated by on-line submission, processing, and dissemination through Science Direct with powerful on-line links to other journals and sources	



4art ARTICLE: INFLUENCE OF ABO LOCUS ON PFA-100 COLLAGEN-ADP CLOSURE TIME IS NOT TOTALLY DEPENDENT ON THE VON WILLEBRAND FACTOR. RESULTS OF A GWAS ON GAIT-2 PROJECT PHENOTYPES.

Núria Pujol-Moix, Angel Martinez-Perez, Maria Sabater-Lleal, Dolors Llobet, Noèlia Vilalta, Anders Hamsten, Joan Carles Souto, José Manuel Soria.

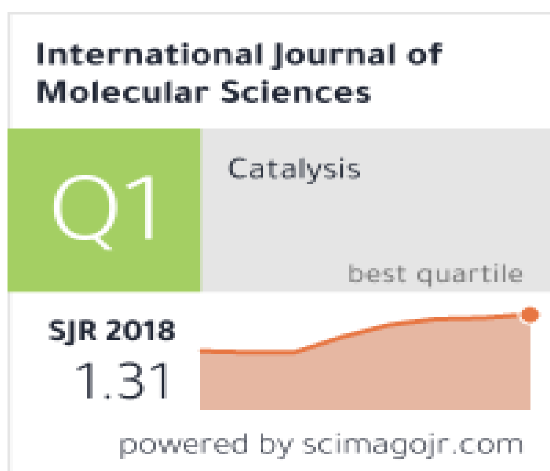
Int. J. Mol. Sci. 2019, 20, 3221; doi:10.3390/ijms20133221

La Doctorand ha realitzat la recerca i recol·lecció de les mostres així com la realització de les tècniques de laboratori corresponents. Ha participat en l'elaboració del manuscrit inicial i en l'aprovació de la versió final.

Descripció de les característiques de la revista **International Journal of Molecular Sciences:**

Country	Switzerland - SIR Ranking of Switzerland	
Subject Area and Category	Biochemistry, Genetics and Molecular Biology Molecular Biology Chemical Engineering Catalysis Chemistry Inorganic Chemistry Organic Chemistry Physical and Theoretical Chemistry Spectroscopy Computer Science Computer Science Applications Medicine Medicine (miscellaneous)	114 H Index
Publisher	Multidisciplinary Digital Publishing Institute (MDPI)	
Publication type	Journals	
ISSN	14220067	
Coverage	2000-ongoing	
Scope	The International Journal of Molecular Sciences (ISSN 1422-0067;	

CODEN: IJMCFK; ISSN 1661-6596 for printed edition) provides an advanced forum for molecular studies in biology and chemistry, with a strong emphasis on molecular biology and molecular medicine. Our aim is to provide rigorous peer review and enable rapid publication of cutting-edge research to educate and inspire the scientific community worldwide. We encourage scientists to publish their experimental, theoretical, and computational results in as much detail as possible, in a regular section (https://www.mdpi.com/journal/ijms/sections) or in a Special Issue (https://www.mdpi.com/journal/ijms/special_issues). Therefore, there is no restriction on the length of the papers or the number of electronic multimedia and supplementary files. For all articles, the full experimental details must be provided so that the results can be reproduced. Electronic files regarding the full details of the experimental procedure, if unable to be published in a normal way, can be deposited as supplementary material (including animated pictures, videos, interactive Excel sheets, software executables and others). Manuscript categories: original scientific research articles, comprehensive reviews, communications, case reports, letters, commentaries, editorials, etc. In the International Journal of Molecular Sciences, molecules are the object of study; among those studies, we find: fundamental theoretical problems of broad interest in biology, chemistry and medicine; breakthrough experimental technical progress of broad interest in biology, chemistry and medicine; and application of the theories and novel technologies to specific experimental studies and calculations



Per tot això els sota signants acrediten l'aportació de la Doctorand en l'elaboració dels articles citats.

CAPÍTOL 4.1

PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK. RESULTS FROM THE RETROVE PROJECT.

CAPÍTOL 4.1.

PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK. RESULTS FROM THE RETROVE PROJECT.

RESUM:

Introducció: La hiperagregabilitat plaquetària i els temps d'oclusió curts del PFA-100® s'han associat a trombosi. El nostre objectiu va ser avaluar si la hiperagregabilitat plaquetària correlacionava amb valors curts de PFA-100® i si s'associava a risc de trombosi venosa en població espanyola.

Material i mètodes: Es van reclutar 400 pacients amb trombosi venosa en el projecte RETROVE (Riesgo de Enfermedad TROmboembólica Venosa). També es van incloure 400 controls sans. Es va determinar l'agregació plaquetària en plasma ric en plaquetes (PRP) per agregometria de transmissió de llum. Es va testar diferents concentracions dels agonistes d'agregació: adenosina difosfat (ADP) i epinefrina (EPI). Es va mesurar el % d'agregació màxima (MA) i el % d'àrea sota la corba (AUC). El risc trombòtic venós associat a hiperagregabilitat plaquetar es va calcular mitjançant regressió logística. Vam estimar la odds ratio (OR) crua i ajustada per edat i sexe.

Resultats: 0,5 µM d'agonista separa els individus amb hipo o hiperagregabilitat plaquetària amb el següent punt de tall:

Per EPI: el percentil 50 per **l'agregació amb 0,5 µM d' EPI (EPI_AUC)** era de 22,53% (>22,53 %= hiper-EPI); en l'anàlisi cru hi havia una diferència estadística entre pacients i controls OR=1,37 (1,03-1,82 IC 95%).

Per ADP: el percentil 75 per l'**agregació amb 0,5 µM d'ADP (ADP_AUC)** era de 29,6% (>29,6%= hiper-ADP). També els pacients eren estadísticament diferents: OR crua= 1,44 (1,05-1,98 IC 95%). Tanmateix, quan la OR de l'agregació d' EPI o ADP es va ajustar per edat aquestes diferències estadístiques van desaparèixer. Les nostres anàlisi indiquen que l'edat sembla incrementar la hiperagregabilitat plaquetària tant en pacients com en controls. EPI_AUC i els valors de PFA-100_EPI correlacionen amb una R=-0,342 ($p<0.01$). Llavors, només el 12% dels valors de PFA-100® són explicats pels valors d'agregació plaquetària.

Conclusions: En el estudi RETROVE, la hiperagregabilitat plaquetària no està associada amb el risc de trombosi venosa.

TITLE: **PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK: RESULTS FROM THE RETROVE PROJECT.**

Blood Coagulation and Fibrinolysis

PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK: RESULTS FROM THE RETROVE PROJECT.

Llobet, Dolors[1]; Vallvé, Cristina[1]; Tirado, Isabel[1]; Vilalta, Noèlia[1]; Carrasco, Marina[1]; Oliver, Artur[2]; Mateo, José[1]; Fontcuberta, Jordi[1]; Souto, Juan Carlos[1].

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PLATELET HYPER-AGGREGABILITY AND VENOUS THROMBOSIS RISK: RESULTS FROM THE RETROVE PROJECT.

Llobet, Dolors[1]; Vallvé, Cristina[1]; Tirado, Isabel[1]; Vilalta, Noèlia[1]; Carrasco, Marina[1]; Oliver, Artur[2]; Mateo, José[1]; Fontcuberta, Jordi[1]; Souto, Juan Carlos[1].
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[2]Haematology Department. Fundació Puigvert. Barcelona. Spain.

ABSTRACT:

Objective: to evaluate if platelet hyper-aggregability correlates with short PFA-100® values and if it is associated with the risk of venous thrombosis (VT) in a Spanish population.

Material and Methods: Patients consisted of 400 individuals with VT recruited for the RETROVE Project (Riesgo de Enfermedad TROmboembólica VEnosa). Also, 400 healthy controls were included. We determined platelet aggregation in Platelet-Rich Plasma (PRP) by light transmission aggregometry. Various concentrations of the aggregation agonists (adenosine diphosphate (ADP) and epinephrine (EPI)) were tested for the % of maximum aggregation (MA) and the % of area under the curve (AUC). The VT risk associated with platelet hyper-aggregometry was calculated by logistic regression. We estimated the crude and adjusted (for sex and age) odds ratio (OR).

Results: 0.5μM of agonist separated both hypo- and hyper-responders with the following cut-off of AUC:

a) For EPI: the 50th percentile for **aggregation with 0.5μM of EPI (EPI_AUC)** was 22.53% (>22.53%=hyper-EPI); from the crude analysis, we found a statistically significant difference between patients and controls OR= 1.37(95%CI: 1.03-1.82).

b) For ADP: the 75th percentile for **aggregation with 0.5μM of ADP (ADP_AUC)** was 29.6% (>29.6%=hyper-ADP). Also, the patients were statistically different: crude OR=1.44(95%CI: 1.05-1.98). Nevertheless, when EPI or ADP aggregation OR was adjusted for age these statistical differences disappeared.

EPI_AUC and PFA-100® values with EPI as agonist correlate with R=-0.342 ($p < 0.01$). Then, only 12% of the PFA-100® values were explained by platelet aggregation.
Conclusion: In the RETROVE study, platelet hyper-aggregability is not associated with VT risk.

Keywords: Platelet hyper-aggregability, platelet reactivity, platelet aggregation, thrombosis, adenosine diphosphate, epinephrine, PFA-100®.

INTRODUCTION:

Sticky Platelet Syndrome (SPS) was described in 1983 [1] as a separate clinical syndrome. It is characterized by an increase of dose-dependent platelet aggregation patterns with adenosine diphosphate (ADP) or epinephrine (EPI) or both. This increase of platelet aggregation is called "platelet hyper-aggregability". Thus, the different dose for testing platelet hyper-aggregability in patients with SPS are: for ADP reagent: 0.58 μM, 1.17 μM and 2.34 μM and for EPI 0.55 μM, 1.1 μM and 11 μM. According to the variable response of platelets to the two agonists, SPS was classified into three types: Type I (platelet hyper-aggregability with both ADP and EPI), Type II (platelet hyper-aggregability only with EPI) and Type III (platelet hyper-aggregability with only ADP). This syndrome was associated with arterial thrombosis such as myocardial infarction and stroke [2-6], also it is associated with recurrent spontaneous abortion [2, 4, 7], chronic kidney disease and kidney graft infarction [8] and venous thrombosis [2, 5, 6, 9]. Antiplatelet therapy is an outstanding clinical response [3].

A number of studies [2-9] of platelet hyper-aggregability referred to SPS, performed with patients with both venous and arterial thrombosis often had few or no controls. These studies included some families and patients of young ages with SPS.

The magnitude of these responses overlaps with normal ranges as reported in population studies [10, 11]. Aggregation of platelets with low doses of EPI and/or ADP in normal people is manifested in two states. One is where platelet aggregation is greater than 60 % (*in vitro* platelet hyper-reactivity) and the other is where aggregation is less than 40% (*in vitro* platelet hypo-reactivity) [10-12]. Few subjects exhibited aggregation in the 40% to 60% range suggesting that less than 40% and more than 60% represent appropriate criteria by which to distinguish between partial or absent aggregation and full or total aggregation.

Yee, *et al.* [11] reported that healthy individuals with platelet hyper-reactivity to EPI also show increased platelet function. They observed that platelet hyper-reactivity rather generalizes to multiple forms of platelet stimulation: from adhesion (agglutination increases after added low dose ristocetin) to activation (after granule release P-selectin expression is increased) to aggregation (aggregation is increased in response to different stimuli). Then, Yee, *et al.* [11] defined hyper-reactive platelet phenotype as a "global" phenomenon in opposition to the classical dose-dependent stimulation of platelets in SPS [13].
Platelet reactivity can be measured using a) a test based on platelet aggregation activated by agonists in Platelet Rich Plasma (PRP) or in whole blood or b) a test for platelet adhesion under shear stress as by PFA-100® analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) [14].

The two tests that measure platelet aggregation use a) light transmission aggregometry (LTA) method when PRP is analyzed and b) impedance aggregometry by Multiplate® analyzer (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) when whole blood is analyzed.

LTA measures platelet aggregation by light transmission through a cell suspension (PRP). When platelets begin to aggregate in response to an agonist, there is a decrease in the turbidity of the suspension with increase light transmission. LTA assesses change in platelet shape, then provides information about platelet activation, adhesion and aggregation. This technique is considered the "gold standard" method. It detects a variety of congenital and acquired disorders of platelet function. The platelets are isolated from the rest of the whole blood and they are under low share rates and only aggregate after

the agonist is added. In this case, they are not expressing under true physiologic conditions.

Whole blood impedance aggregometry (by Multiplate® analyzer) measures the attachment of platelets to platinum electrodes, resulting in an increase of electrical resistance between the electrodes. The change of impedance is proportional to the amount of platelets that stick to the electrodes. Whole blood impedance aggregometry tries to reflect platelet function *in vivo* produced on surfaces. It is more physiological than LTA, but it does not assess changes in platelet shape. It has more interference depending on the quality of blood (hemolyzed blood, lipemic blood). It provides quality and quantitative data on aggregation response, but some studies [15, 16] suggested that it is inferior to LTA for detecting abnormalities from platelet disorders. The platelet aggregation response to antiplatelet medication is well monotorized by whole blood impedance aggregometry.

PFA-100® simulates vessel wall by physiological stress conditions. It contains a membrane coated with collagen and ADP or EPI also. This membrane has a hole through which anticoagulated blood can pass. The presence of this agonists and high share rates results in platelet adhesion, activation and aggregation and finally the platelet plug is formed and the hole is covered. The closure time (CT) is measured in seconds to cover the hole. This time is inversely proportional to the capacity of platelet function. It is influenced by von Willebrand levels, ABO blood group, hematocrit and platelet count. We refer PFA-100® analyzer as an analyzer that measures global platelet function tests (in terms of platelet adhesion and platelet aggregation) in contrast to LTA that measures platelet aggregation.

PFA-100® method screens mostly bleeding disorders. In this setting, PFA-100® is highly sensitive and generally highly sensitive methods, it often gives false positive results due to its high sensitivity. Recently, we reported [17] that short CT with the agonists ADP and EPI using a PFA-100® analyzer (PFA_ADP and PFA_EPI) are associated with the risk of venous thrombosis. The co-joint risk from PFA_EPI and PFA_ADP, showed the strongest association with venous thrombosis risk. In addition, the PFA-100® analyzer is useful in an *in vitro* assay for evaluating overall platelet function [18-19].

In our study, we tested platelet reactivity in terms of platelet aggregation, thus, we refer to platelet hyper or hypo- aggregability.

Platelet hyper-aggregability has been associated with arterial thrombosis such as myocardial infarction and stroke [2-6, 20-24] and venous thromboembolism [2, 9] but in contrast, in the Framingham Heart Study [25], platelet aggregability was only weakly associated with venous thrombosis. Also, platelet aggregation in whole blood was not associated with the risk of venous thrombosis [17].

The aim of our study was to determine if platelet hyper-aggregability correlates with short PFA-100® values and if it is associated with the risk of venous thrombosis in a Spanish population.

MATERIALS AND METHODS:

Study Design

The RETROVE Study (Riesgo de Enfermedad TROmboembólica VEnosa): is a case- control study with patients who suffer from venous thrombotic disease; its objective was to define a risk algorithm, which includes clinical, plasmatic, genetic and epigenetic data. This algorithm should evaluate the individual's risk of venous thrombosis through a score of thrombogenicity. Individuals were recruited at the Hospital de la Santa Creu i Sant Pau, Barcelona (Spain) between 2012 and 2016. It included 400 patients (≥18 years) with thrombosis with no cancer and no liver or renal dysfunction. Also, 400 healthy individuals were included as controls. They were distributed according to age and sex in a Spanish population (2001 census). The controls were not matched intentionally for sex or for age. Thus, any statistical inference must be adjusted for these variables. Detailed population baseline characteristics are shown in Table 1.

Doppler ultrasonography, tomography, magnetic resonance, arteriography, phlebography and pulmonary gammagraphy were used to diagnosis thrombosis. Table 2 lists the classification of unprovoked or spontaneous thrombotic event or provoked or non–spontaneous thrombotic event [26, 27]. The provoking factors were (one or more within three months previous to an event): surgery, immobilization, pregnancy or puerperium, oral contraceptives, prothrombotic non–neoplastic diseases and other circumstances.

Our study was conducted in accordance with the Declaration of Helsinki. All procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau in Barcelona, Spain. Written informed consent was obtained from all participants

Blood Collection

Blood was collected 6 months after the most recent thrombotic event. Also, blood was collected 3 months after an acute event as trauma, inflammation or sepsis. Antivitamin K treatment was withdrawn and the blood samples were taken after a washout period of at least 20 days. Blood samples were collected from the antecubital vein and immediately anticoagulated with 1/10 volume of 0.129M sodium citrate (BD Vacutainer Becton, Dickinson and Company, New Jersey, USA). Platelet-Rich Plasma (PRP) was obtained by centrifugation at 160 g for 10 min. To minimize the interference in the analysis of platelet function none of the individuals had any excessive physical activity 24 hours before the analyzing platelet function. Also, none of the individuals had taken aspirin, NSAID (non-steroidal anti-inflammatory drugs), phosphodiesterase inhibitors, desmopressin, antibiotics, chemotherapeutics or plasma expanders in the 10 days before blood extraction. Platelet-Poor Plasma (PPP) was obtained by centrifugation at 2000 g for 20 min. PPP was frozen and stored at – 40°C until fibrinogen was analyzed. To measure whole blood impedance aggregometry (by Multiplate® analyzer), samples were collected in Hirudin at 25 µg/ml (Roche Diagnostics GmbH, Mannheim, Germany). Without exception, PFA-100® test and platelet aggregation were analyzed between 30 minutes and two hours after extraction.

Determination of fibrinogen.

Fibrinogen was determined by the Clauss method in the STA®-R analyzer (Diagnostica Stago, Ansières, France) according to the manufacturer's instruction.

Determination of PFA-100® values as a test of Global Platelet Function:

Global platelet function was measured as closure time (CT) with the agonists ADP and EPI using a PFA-100® analyzer (Platelet Function Analyzer-100). For the PFA analysis, the citrated whole blood sample was transferred to the reservoir of the disposable test cartridges (PFA_ADP and PFA_EPI), already inserted in the instrument, and both closure times were recorded. Normal reference ranges were from 58 to 123 seconds, for the PFA_ADP and, from 72 to 191 seconds, for the PFA_EPI.

Determination of Platelet Aggregation:

<u>1.- Aggregation in PRP by LTA method:</u>

PRP was adjusted to a concentration of 250 x10^9 platelets/L [28] to diminish the potential bias among individuals. We used two agonists: ADP and EPI to test platelet aggregation with PRP. For this test, we used a four-channel light transmission aggregometer (LTA) (Biometa, Helena) to measure platelet aggregation. It measured the % of maximum aggregation (MA) and the % of area under the curve (AUC).

First, different concentrations of each agonist were tested to obtain the concentration that distinguished hyper- and hypo-responders: we tested aggregation using a final concentration of 2 µM, 1 µM, 0.5 µM and 0.25 µM for ADP and 10 µM, 1 µM, 0.5 µM and 0.12 µM for EPI.

The agonists were diluted with physiological saline. Finally, we used ADP and EPI at 0.5µM to separate hyper- and hypo-responders and the higher concentrations (2 µM for ADP and 10 µM for EPI) as a positive control. Hyper- and hypo-responders were characterized as previously described [10, 12]: individuals who responded ≥60% MA were considered hyper-responders for ADP (hyper-ADP) or for EPI (hyper-EPI). Individuals who failed to aggregate (≤ 40% MA) were considered hypo-responders for ADP (hypo-ADP) or for EPI (hypo-EPI). Hypo-responders failed to aggregate at 0.5 µM and lower concentrations.

Platelet aggregation was measured twice in the 50-first hyper-responders obtained in 2012.

2.- Aggregation in Whole Blood by impedance method:

We measured platelet aggregation with whole blood in response to ADP. We used an analyzer that uses multiple electrode aggregometry: Multiplate® analyzer (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) to measure platelet aggregation. Whole blood was added to the test cuvettes, stirred and warmed to 37 °C. This test measured the % of maximum aggregation (MA) and the % of area under the curve (AUC).

We tested aggregation at 2 µM, 1 µM, 0.5 µM and 0.25 µM final concentrations of ADP. Neither of these concentrations separated hypo or hyper-responders as previously described [10, 12].

Statistics Analyses:

Spearman's coefficient correlation was used to calculate values between PFA-100® and aggregation in PRP (measuring by MA and AUC). In aggregation with PRP by LTA method: Results were expressed in median differences because they did not follow a normal distribution, kolmogorov test was applied. 50th percentile of AUC for EPI and 75th percentile of AUC for ADP were calculated in controls to discriminate hypo- or hyper-responders. Frequencies between hyper- and hypo-responders were compared using the χ^2 test. p values < 0.05 were considered statistically significant. A conditional logistic regression method was used to estimate both the crude and adjusted odds ratio (OR) with 95% confidence intervals. According to previous studies [29, 30] there are potential confounding factors that can introduce bias in determining the risk factors for thrombosis. So, we analyzed the venous thrombosis risk using three statistical models. The venous thrombosis risk models were 1) unadjusted or crude (Model 1); 2) adjusted by sex (Model 2) and 3) adjusted by sex and age (Model 3). Differences between PRP aggregation and whole blood impedance aggregometry were calculated by the Wilcoxon test. An ANOVA by two factors was applied for fibrinogen differences related to platelet aggregability (hypo- and hyper-responders) by individual group (patients and controls). Also, an

ANOVA by two factors was applied for PFA-100® differences related to platelet aggregability by individual group.

RESULTS:

1.- Aggregation in PRP (by LTA method):

We found that 0.5 µM with both agonists (EPI and ADP) clearly separated the two populations, (ie, hyper- and hypo-responders) according to the criteria proposed previously [10,12]. The results are shown in Figure 1.

Aggregation (MA) with EPI at 0.5 µM:

Thirty-eight % of the patients were hypo-responders and 53% were hyper-responders. In the control group: 45% were hypo-responders and 48% were hyper-responders. 9% for patients and 7% for controls were between 40-60% MA. These differences were not statistically significant.

Aggregation (MA) with ADP at 0.5 µM:

After treatment with ADP, sixty-nine % of the patients were hypo-responders and 24% were hyper-responders. In the control group: 74% were hypo-responders and 22% were hyper-responders. Also, 7% of patients and 4% of controls were between 40-60% MA. These differences were not statistically significant.

Aggregation (AUC) with EPI at 0.5 µM (EPI_AUC):

Significant median differences of EPI_AUC levels were found between patients and controls (EPI_AUC: 27.01% AUC vs 22.53% AUC respectively; p=0.014).

Aggregation (AUC) with ADP at 0.5 µM (ADP_AUC):

Significant median differences of ADP_AUC levels were found between patients and controls (ADP_AUC: 19.10 % AUC vs 12.92 % AUC respectively; p=0.001).
The following percentiles: 5th, 10th, 25th, 50th, 75th, 90th, 95th for aggregation (AUC) were found for the patients and controls with both agonists as shown in

Table 3. We observed higher statistically significant values in patients than in the controls with both agonists except in 95th percentile of ADP.

The cut-off of AUC that separated hyper- and hypo-responders was 50th percentile (<22.53%) for EPI and for ADP: 75th percentile (<29.59%) (Figure 2).

A statistically significant difference was found between patients and controls:

Aggregation with EPI at 0.5µM (EPI_AUC) showed a statistically significant difference with 50th percentile (>22.53 %= hyper-EPI): which was higher in patients than in controls (57.4% vs 49.6%; difference of 7.8%; CI95%:1.032-1.82) (p=0.031). The associated thrombotic OR was 1.37 (1.03-1.82 CI95%). When OR was adjusted for sex alone, the OR was maintained, but when it was adjusted for sex and age, the statistical significance was lost 1.17 (0.83-1.64 IC95%) (ns) (Table 4).

Aggregation with ADP at 0.5µM (ADP_AUC) also showed a statistically significant difference with 75th percentile (>29.59 %=hyper-ADP): a higher percentage in patients than in controls was observed (32.7% vs 25.2%; difference of 7.5%; CI95%:1.05-1.97) (p=0.032). The associated thrombotic OR was 1.44 (1.05-1.98 95%IC). When adjusted for sex only the OR was retained but when adjusted for both sex and age the statistical significance was lost (OR=1.14 (0.84-1.55 IC 95%) (ns) (Table 4).
It was co-joint both hypo-responders (hypo-ADP and hypo-EPI) as a total hypo-responders and also it was co-joint hyper-ADP and/or hyper-EPI as total hyper-responders. When total hyper-responders were measured, the OR obtained was 1.46 (52.2% in patients vs 44.8% in controls; difference of 7.4% CI 95%: 1.04-2.04) (p=0.032) but again when it was adjusted for sex and age there was no statistical significance (OR=1.06 (0.73-1.15)) (ns).

It is notable that significant median differences of ADP_AUC levels and EPI_AUC levels were found between women and men (ADP_AUC: 27.3 % AUC vs 20.0 % AUC; p=0.01) and by EPI_AUC: 31.6 % AUC vs 26.4 % AUC; (p=0.001). Instead, the ADP_AUC and EPI_AUC levels were higher in women. This significant difference was lost when adjusted for age in ADP_AUC and EPI_AUC. Age correlated positively with platelet aggregability using both agonists.

Age was stratified into three ranges: young (18-35 years), middle (36-60 years) and elderly (>60 years) and platelet aggregability was evaluated. As is shown in Table 5, platelet aggregability seems to increase with age in patients and in controls.

The levels of fibrinogen in hypo- and hyper-responders were evaluated:

As is shown in Figure 3, fibrinogen levels were higher in total hyper-responders than in total hypo-responders (in controls 3.54g/L vs 3.26 g/L; in patients 3.94g/L vs 3.61 g/L) (p= 0.0001). When fibrinogen levels were evaluated in relationship to only hyper/hypo-EPI or with hyper/hypo-ADP the behavior was the same (results not shown).

2.- Aggregation in Whole Blood (by an impedance method):

The concentrations of ADP (2 μM or 1 μM or 0.5 μM or 0.25 μM at final concentrations) did not separate the hypo- and hyper-ADP. Figure 4 shows the aggregation at 0.5 μM of ADP in patients and in controls.

No differences between patients and controls were found in MA or AUC in aggregation in whole blood. Table 6 shows the results of the median comparison (both in PRP and in whole blood).

As we previously described [17], no association was found between aggregation in whole blood and the risk of venous thrombosis.

3.- PFA-100® values as a test of Global Platelet Function:

We studied the relationship between aggregation and global platelet function. We found a significant negative correlation between the AUC aggregation in PRP with low doses of agonist (as ADP as EPI) and PFA-100® values with EPI as agonist (PFA_EPI): -0.322 and -0.342 ($p<0.001$) respectively. Less correlation was found between PRP aggregation and whole blood aggregation. The strongest correlations were found between EPI and ADP aggregation in PRP. Table 7 lists the values of the statistically significant correlations.

The mean PFA-100® values in hypo and hyper-responders were compared:

For PFA_EPI: CT levels were lower in total hyper-responders than in total hypo-responders (in controls: 122s vs 131s; in patients: 105s vs 115s) (p=0.0001) (Figure 5). When PFA_EPI values were evaluated in comparison to only hyper/hypo-EPI or to hyper/hypo-ADP the results were the same (results not shown).

For PFA_ADP: No statistically significant differences were found between CT levels and hyper- or hypo-responders both in the controls and the patients (Figure 5).

DISCUSSION:

We studied if platelet hyper-aggregability was associated with venous thrombosis. Our subjects were studied for *in vitro* platelet hyper-aggregability. We found that 0.5μM of agonist separated both hypo- and hyper- responders. Platelet hyper-responders were found in both patients and controls. Our results agree with those reported by Yee, *et al.* [10, 11] who defined hypo-responders as individuals who had a maximal aggregation <40% and hyper-responders with aggregation >60% with the same low concentration of agonist. We analyzed the behavior of both agonists (ADP and EPI) in terms of MA and AUC (Figure 1 and 2 and Table 4). We obtained a cut-off with each agonist which separated the two populations (hypo- and hyper-responders) (Figure 2). MA measures were defined as binary, "all or none" type of aggregation response. Interestingly, in contrast, when AUC was measured, this binary trait was transformed into a quantitative continuous trait and bimodal distribution was maintained.

Our results suggest that platelet hyper- and hypo-aggregability is not significantly different in the control than in the patients. We found statistically significant crude OR between patients and controls in platelet aggregability measuring by AUC. This could mean that patients had a faster response to the agonist than the controls. However, when OR was adjusted for age the significance was lost. This could mean that platelet aggregability depends on age for thrombosis association. Age seems to increase platelet aggregability both in patients and in controls (Table 5). Favaloro [31] reported that platelet activation increases with age because there are age-related mechanisms that activate platelets: oxidative stress, increases phospholipids membrane content,

increase platelet-monocyte interaction and menopause. Thus, the increases in platelet activation could contribute to prothrombotic phenotype observed in an aged population.

The relationship between platelet hyper-aggregability and venous thrombosis is controversial. No association was found in the Framingham Heart Study [25] or in platelet whole blood aggregation parameters measured by Multiplate® analyzer [17].

In contrast, other studies [2, 5, 6, 9, 32, 33] found an association between platelet hyper-aggregability, measured with PRP by LTA method, and venous thrombosis. In detail: in the Tekgündüz, *et al*. study [33] controls were not included. In the Hayes, *et al.* study [9] platelet hyper-aggregability with three different agonists (ADP, EPI and arachidonic acid) were analyzed. They found association only between platelet hyper-aggregability induced by EPI and venous thrombosis. Also, they did not find a correlation between platelet hyper-aggregability and PFA-100® values. In the Weber, *et al.* study [32], platelet hyper-aggregability was analyzed with 4 agonists (thrombin receptor-activating peptide (TRAP-6), collagen, ADP and EPI). They found an association between venous thrombosis and platelet hyper-aggregability when it was induced by collagen and TRAP-6, not with ADP or EPI.

There are some studies that relate to platelet hyper-aggregability measured by whole blood aggregation. As describe above, our group did not find an association between platelet whole blood aggregation parameters and venous thrombosis [17]. In contrast, Campello, *et al.* detected hypercoagulability in whole blood thromboelastometry (ROTEM®) and impedance aggregometry (Multiplate®) in obese patients [34]. The platelet aggregation response to antiplatelet medication is well monotorized in whole blood impedance aggregometry. Some studies [35-36] measure the response to antiplatelet drugs measured by Multiplate® analyzer in patients with arterial thrombosis (ischemic stroke or transient ischemic attack). They found that individual non-responders to clopidogrel had higher platelet aggregation response than individuals who responded to clopidogrel after stimulation with arachidonic acid or thrombin receptor-activating peptide (TRAP) indicating a general platelet hyper-reactivity.

In patients with bleeding problems, or in preoperative patients, Multiplate® and PFA-100® did not discriminate between patients with and without platelet function disorders and aggregation curve abnormalities as detected by LTA. But, they detected Glanzmann Thrombastenia. This means that they can detect severe but not mild platelet dysfunction [15]. Some studies [16] of aggregation using whole blood aggregometry instruments have shown that the method is inferior to LTA for detecting abnormalities of platelets disorders.

Figure 3 shows that the Multiplate® analyzer did not distinguish between hypo- and hyper-responders, possibly because the plot of aggregation is less sensitive or that the concentrations are not adequate. Hayward [16] explained that whole blood aggregometry results are difficult to compare to LTA findings as the agonist concentrations are different; then she suggested that the concentration required to measure aggregation with ADP in whole blood impedance aggregometry must be 5 times higher as measure by LTA. Thus, it could be that 0.5 µM was not a suitable concentration.

Moreover, in Multiplate® analyzer, we tested aggregation only with ADP because EPI did not show a response in whole blood. This result agrees with Hayward [16] who described some agonists (e.g. epinephrine) that were useful for diagnosing platelet disorders by LTA were not suitable for whole blood aggregometry as there was greater variability in weak agonist responses with whole blood aggregometry. Also it could explain the discrepancy about PRP and whole blood results and hyper-aggregability. Many patients show platelet hyper-aggregability only after the addition of low concentrations of EPI. But, whole blood impedance aggregometry does not detect changes in platelet shape. Sokol, *et al.* [37], defined some rules for evaluating platelet hyper-aggregability. They recommended the use of only LTA. We did not find an association between whole blood aggregation and venous thrombosis risk.

Recently, our group reported [17] that short closure time (CT) values in PFA-100® were related to the risk of venous thrombosis. We studied the relationship between the results of aggregation test and the global platelet function test. We obtained a negative correlation between them (R=-0.342). This suggests that only 12% (R^2) of PFA-100® values are due to aggregation with low doses of agonist (Table 3). Hyper-responders to platelet aggregability had lower PFA_EPI levels than hypo-responders. This result agrees with Yee, *et al*. [11], but this

behavior is the same in patients and in controls. With PFA_ADP, no significant results were obtained (Figure 4).

Hyper-responders to platelet aggregability had higher levels of fibrinogen than hypo-responders as Yee, *et al*. [10] described, but this behavior was the same in controls and in patients (Figure 3).

Our study described hyper and hypo responders to platelet aggregability. It did not study individuals with or without Sticky Platelet Syndrome (SPS). Platelet aggregability is affected greatly by pre-analytical issues [37], so SPS diagnostic needs to be repeated, if the results of LTA are abnormal, within 4 or 6 weeks. We obtained a reproducibility of platelet aggregation test of 85% with the 50 first hyper- responders analyzed twice (data not shown).

It is noteworthy that we found that platelet hyper-aggregability (with both agonists) is associated with venous thrombotic risk in women, but not in men. However, when we adjusted for age the significant differences between the sexes was lost confirming that age is a confounding factor on platelet hyper-aggregability.
Age seems to increase platelet hyper-aggregability; it would be interesting to do clinical assays with an aged population with or without antiplatelet agents. Maybe, patients with short CT of PFA-100® might profit from treatment with platelet aggregation inhibitors.

Our study has some limitations. One is that we analyzed platelet aggregometry using PRP adjusted to 250 $x10^9$ platelets/L to diminish the potential bias between patients. The use of PRP adjusted or not to measure platelet aggregation is controversial in LTA. Castilloux *et al* [38], found that the use of PRP adjusted is superior to PRP non adjusted for detecting bleeding disorders, but no standard rules exist for thrombotic platelet disorders. When PPP was added to PRP to standardized platelet count decreases maximal aggregation, especially when a weak agonist is added to measure platelet aggregation (as EPI and ADP) [39]. This suggests that in our study there was less hyper-responders. The previous studies were done with PRP adjusted [1, 9, 10, 12, 33]. Another limitation is the possibility that our patients had a higher thrombotic risk than a random group of patients because we are a reference center in our region. In addition, since all of the platelet parameters were

measured at least 6 months after the thrombotic event to avoid acute phase, we do not know if our results would have been different if we studied patients immediately or shortly after the thrombotic event. Finally, in the RETROVE Study patients and controls were not matched for age and sex, but our patients were older than the controls.

Conclusions:

Platelet hyper-aggregability is not independently associated with the risk of venous thrombosis. The correlation between platelet hyper-aggregability and global platelet function tests, measured by means of PFA-100® is modest, since only 12 % of the PFA-100® value is estimated due to aggregation with low concentration agonists.

The concentration of agonist (ADP or EPI) that separated clearly hyper- and hypo- responders was 0.5µM at final concentration in PRP aggregation. Platelet hyper-aggregability seems to increase with age, in the same way in both patients and in controls.

Platelet hyper-aggregability is related to high fibrinogen levels and low PFA-100® values, but this behavior is the same in both patients and in controls.

Recently, several studies [17, 40, 41] have implicated platelet function and platelet phenotypes in the pathophysiology of venous thrombosis. Nevertheless, our results confirm that platelet aggregability and platelet hyper-aggregability are not clearly related to the risk of venous thrombosis. Further studies, that examine platelet adhesiveness, might clarify the role of platelets in the risk of venous thrombosis.

Acknowledgements

We would like to thank Professor William Stone and Dr. Montserrat Borrell for reviewing the manuscript.

Sources of Funding

Spanish grants: FIS PI12/00612 and FIS PI 15/0269

Conflict of Interest

The authors report no conflicts of interest.

REFERENCES

1. Holiday PL, Mammem E, Gilroy J. Sticky platelet syndrome and cerebral infarction in young adults. Paper presented at: The Ninth International joint Conference on Stroke and Cerebral Circulation 1983; Phoenix, AZ.USA.
2. Kubisz P, Stasko J, Holly P. Sticky platelet syndrome. Semin. Thromb.Hemost 2013; 39: 674-683.
3. Al-Mefty O, Marano G, Rajaraman S, Nugent GR, Rodman N. Transient ischemic attacks due to increased platelet aggregation and adhesiveness. Ultrastructural and function correlation. J Neurosurg. 1979; 50: 449-453.
4. Bick RL, Sticky platelet syndrome: A common cause of unexplained arterial and venous thrombosis. Clin Appl Thromb/Hemost 1998;4(2): 77-81.
5. Mammen EF. Ten years´experience with the Sticky platelet syndrome. Clin Appl Thromb/Hemost. 1995;1 (1):66-72.
6. Frenkel EP, Mammen EF. Sticky platelet syndrome and thrombocythemia. Hematol Oncol Clin N Am. 2003; 17: 63-83.
7. Bick RL. Recurrent miscarriage syndrome due to blood coagulation protein/platelet defects: prevalence, treatment and outcome results. DRW Metroplex Recurrent Miscarriage Syndrome Cooperative Group. Clin Appl Thromb Hemost. 2000; 6(3): 115-25.
8. Mühlfeld AS, Ketteler M, Schwamborn K, et al. Sticky platelet syndrome: an underrecognized cause of graft dysfunction and thromboembolic complications in renal transplant recipients. Am J Transplant. 2007; 7 (7): 1865-68.
9. Hayes C, Kitahara S, Tcherniantchouk O. Decreased threshold of aggregation to low-dose epinephrine is evidence of platelet hyperaggregability in patients with thrombosis. Hematol Rep 2002; 6: 325-328.

10. Yee DL, Sun CW, Bergeron AL, Dong J-F, Bray PF. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 2005; 106: 2723-2729.
11. Yee DL, Bergeron AL, Sun CW, Dong J-F, Bray PF. Platelet hyperreactivity generalizes to multiple forms of stimulation. J Thromb Haemost 2006; 4: 2043-50.
12. Berger J.S, Becker R.C, Kuhn C, Helms M.J, Ortel T.L, Williams R. Hyperrreactive platelet phenotypes: Relationship to altered serotonin transporter number, transport kinetics and intrinsic response to adrenergic co-stimulation. Thromb Haemost 2013 ;109 : 85-92.
13. Mammen EF. Sticky platelet syndrome. Semin Thromb Hemost. 1999; 25(4): 361-65.
14. Pujol-Moix N, Martinez-Perez A, Sabater-Lleal M, Llobet D, Vilalta N, Hamsten A, Souto JC, Soria JM. Influence of ABO Locus on PFA-100 Collagen-ADP Closure Time Is Not Totally Dependent on the Von Willebrand Factor. Results of a GWAS on GAIT-2 Project phenotypes. Int. J. Mol. Sci. **2019**, 20, 3221; doi:10.3390/ijms20133221
15. Floor CJI Moenen, Minka JA Vries, PJ Nelemans, KJM van Rooy, JRRA Vranken , PWM Verhezen, RJH Wetzels, H ten Cate, HC Schouten, EAM Beckers, YMC Henskens. Sreening for platelet function disorders with Multiplate and platelet function analyzer. Platelets, 2019; 30: 1, 81-87.
16. Catherine P.M. Hayward. Practical manual: Platelets. Chapter: Laboratory diagnosis of platelet disorders.
17. Vázquez-Santiago M, Vilalta N, Cuevas B, Murillo J, Llobet D, Macho R, Pujol-Moix N, Carrasco M, Mateo J, Fontcuberta J, Soria JM, Souto JC. Short closure time values in PFA-100 are related to venous thrombotic risk. Results from the RETROVE Study. Thromb Research 2018; 169: 57-63.
18. Jilma B. Platelet function analyzer (PFA-100): a tool to quantify congenital or acquired platelet dysfunction. J.Lab. Clin. Med. 2001; 138 152-163.
19. Favaloro EJ. Clinical utility of the PFA-100. Semin Thromb Hemost 2008; 34: 709-733.
20. Trip MD, Cats VM, van Capelle FJ, Vreeken J. Platelet hyperreactivity and prognosis in survivors of myocardial infarction. N Engl J Med. 1990;322:1549-1554.
21. Kabbani SS, Watkins MW, Ashikaga T et al. Platelet reactivity characterized prospectively: a determinant outcome 90 days after percutaneous coronary intervention. Circulation 2001; 104: 181-186

22. Kabbani SS, Watkins MW, Ashikaga T, et al Usefulness of platelet reactivity before percutaneous coronary intervention in determining cardiac risk one year later. Am J Cardiol 2003; 91: 876-878.
23. Konstantopoulos K, Grotta JC, Sills C, Wu KK, Hellums JC. Shear-induced platelet aggregation in normal subjects and stroke patients. Thromb Haemost 1995; 74: 1329-1334.
24. Vanschoonbeek K, Feijge MA, Keuren JF, et al. Thrombin-induced hyperactivity of platelets of young stroke patients: involvement of thrombin receptors in the subject-dependent variability in Ca 2+ signal generation. Thromb Haemost 2002; 88: 931-937.
25. Puurunen MK, Hwang S-J, O'Donnell CJ, Tofler G, Johnson AD. Platelet function as a risk for venous thromboembolism in the Framingham Heart Study. Thromb Research 2017; 15157-62.
26. Martinez C, Cohen AT, Bamber L, Rietbrock S, Epidemiology of first and recurrent venous thromboembolism: a population-based cohort study in patients without active cancer. Thromb. Haemost 2014; 112 : 255–63. doi:10.1160/TH13-09-0793.
27. Kearon C, Ageno W, Cannegieter SC, Cosmi B, Geersing G-J, Kyrle PA, and P. and D.V. in T.D. Subcommittees on Control of Anticoagulation, Categorization of patients as having provoked or unprovoked venous thromboembolism: guidance from the SSC of ISTH., J Thromb Haemost 2016; 14 : 1480–3. doi:10.1111/jth.13336
28. Ledford-Kraemer M. Standardization of Platelet Function Testing CLSI Guideline H58-P Platelet Function Testing by Aggregometry. MBA, BS, MT (ASCP) SH, CLOT-ED, Inc.
29. Prandoni P, Venous and arterial thrombosis: two aspects of the same disease? Eur J Intern Med 2009; 20 : 660-661.
30. Previtali E, Bucciarelli P, Passamonti SM, Martinelli I, Risk factors for venous and arterial thrombosis. Blood Transfus 2011; 9 : 120-138.
31. Favaloro EJ, Franchini M, Lippi G. Aging Hemostasis: Changes to laboratory Markers of Hemostasis As We Age-A Narrative Review. Semin Thromb Hemost 2014; 40: 621-633.
32. Weber M, Gerdsen F, Guttensohn K, Schoder V, Eifrig B, Hossfeld. DK Enhanced platelet aggregation with TRAP-6 and collagen in platelet aggregometry in patients with venous thromboembolism. Thromb Res 2002; 107 : 325-328 , https://doi.org/10.1016/S0049-3848(02)00351-1
33. Tekgunduz E, Demir M, Akyol Erikci A, Akpinar S, Ozturk E, Kirkizlar O. Sticky platelet syndrome in patients with uninduced venous thrombosis. Turk J Hematol 2011; 30: 48-52. https://doi.org/10.5152/tjh2011.79.

34. Campello E, Spieza L, Zabeo E, Maggiolo S, Vettor R, Simioni P. Hypercoagulability detected by whole blood thromboelastometry (ROTEM®) and impedance aggregometry (MULTIPLATE®) in obese patients. Thromb Res, 2015; 135: 548-53.
35. Lundström A, Wallén H, von Arbin M, Jörneskog G, Gigante B, Dembrower KH, Laurencikas E, Laska AC. Clopidogrel resistance after minor ischemic stroke or transient ischemic attack is associated with radiological cerebral small-vessel disease. J Stroke Cerebrovasc Dis, 2015; 24, 2348-57.
36. Lundström A, Laska AC, von Arbin M, Jörneskog G, Wallén H. Glucose intolerance and insulin resistance as predictors of low platelet response to Clopidogrel in patients with minor ischemic stroke or TIA. Platelets, 2014; 25, 102-10.
37. Sokol J, Skerenova M, Jedinakova Z, Simurda T, Skonorva I, Stasko J, Kubitz P. Progress in the understanding of Sticky Platelet Syndrome. Semin Thromb Hemost. DOI http://dx.doi.org/10.1055/s-0036-1584352.
38. Castilloux J.F.; Moffat K A; Liu Y; Seecharan J; Pai M; Hayward C.P.M. A prospective cohort study of light transmission platelet aggregometry for bleeding disorders: Is testing native platelet-rich plasma non-inferior to testing platelet count adjusted samples? Thrombosis and Haemostasis 2011; 106.4
39. Mani H, Luxembourg B, Kläffling C, Erbe M, Lindhoff-Last E. Use of native or platelet count adjusted platelet rich plasma for platelet aggregation measurements. J Clin Pathol 2005; 58:747–750. doi: 10.1136/jcp.2004.022129.
40. Vázquez-Santiago M, Vilalta N, Ziyatdinov A, Cuevas B, Macho R, Pujol-Moix N, Carrasco M, Mateo J, Fontcuberta J, Soria JM, Souto JC. Platelet count and plateletcrit are associated with an increased risk of venous thrombosis in females. Results from the RETROVE study. Thromb Res. 2017 Sep; 157:162-164. doi: 10.1016/j.thromres.2017.07.022. Epub 2017 Jul 23
41. Llobet D, Vallve C, Tirado I,Vilalta N, Murillo J, Cuevas B, Roman L, Carrasco M, Oliver A, Mateo J, Fontcuberta J,Souto JC. VAMP8 and serotonin transporter levels are associated with venous thrombosis risk in a Spanish female population. Results from the RETROVE Project. Thromb Res 2019; 181: 99–105, doi.org/10.1016/j.thromres.2019.07.023

Table 1: Clinical characteristics of patients and controls in the RETROVE Study.

Baseline characteristics*	Patients		Controls		p†
	Men	Women	Men	Women	
Gender (n)	196	204	194	206	
Age (years) (mean ± SD)	61.5 ± 16.9	66.2 ± 20.0	47.6 ± 17.7	50.5 ± 18.7	$< 2.2 \cdot 10^{-16}$
BMI ($kg \cdot m^{-2}$) (mean ± SD)	28.0 ± 4.1	28.3 ± 6.1	26.0 ± 25.4	24.4 ± 4.1	$< 2.2 \cdot 10^{-16}$
Smokers (n, %)	37 (18.9)	18 (8.8)	40 (20.6)	35 (17.0)	NS
Alcohol consumption (n, %)	119 (60.7)	76 (34.3)	130 (67.0)	98 (47.6)	0.023
Hypertension (n, %)	83 (42.3)	94 (46.1)	35 (18.0)	40 (19.4)	$8.27 \cdot 10^{-15}$
Dyslipidaemia (n, %)	62 (31.6)	75 (36.8)	36 (18.6)	43 (20.9)	$3.46 \cdot 10^{-6}$
Use of Statins (n, %)	46 (23.5)	60 (29.4)	18 (9.3)	32 (15.5)	$5.81 \cdot 10^{-7}$
Diabetes mellitus (n, %)	23 (11.7)	17 (8.3)	10 (5.2)	10 (4.9)	0.0073
Autoimmune disease (n, %)	20 (10.2)	21 (10.3)	14 (7.2)	18 (8.7)	NS
Ictus haemorrhagic (n, %)	0 (0.0)	2 (1.0)	1 (5.0)	0 (0.0)	NS
Arterial thrombosis background (n, %)	14 (7.1)	15 (7.4)	1 (5.0)	1 (5.0)	$1.08 \cdot 10^{-6}$
Non–steroidal anti–inflammatory drugs (n, %)	29 (14.8)	58 (28.4)	23 (11.9)	34 (16.5)	0.0058
Anti–platelet drugs (n, %)	24 (12.2)	18 (8.8)	2 (1.0)	5 (2.4)	$2.46 \cdot 10^{-7}$

(*) Number of individuals in each group. The percentages are in parentheses. Individuals excluded for technical reasons are: 2 individuals for the BMI, 10 for the ictus haemorrhagic and 10 for the arterial thrombosis background.

(†) The statistical differences, fixed in a $p \leq 0.05$, are reported only for descriptive purposes. The non–significant results are listed as NS.

Table 2: Characteristics of 400 consecutives thrombotic events in patients in the RETROVE Study.

	Spontaneous			Non–spontaneous		
	M*	**W***	**Total**†	**M***	**W***	**Total**†
Isolated deep vein thrombosis	82 (56.2)	63 (49.6)	145 (53.1)	27 (54.0)	38 (49.4)	65 (51.2)
No isolated deep vein thrombosis	30 (20.6)	19 (15.0)	49 (18.0)	10 (20.0)	11 (14.3)	21 (16.5)
Isolated pulmonary embolism	31 (21.2)	42 (33.1)	73 (26.7)	12 (24.0)	26 (33.8)	38 (29.9)
Visceral thrombosis	2 (1.4)	–	2 (0.7)	1 (2.0)	–	1 (0.8)
Venous sinus thrombosis	1 (0.7)	3 (2.4)	4 (1.5)	–	2 (2.6)	2 (1.6)
Total‡	146 (36.5)	127 (31.8)	273 (68.3)	50 (12.5)	77 (19.3)	127 (31.8)

(*) The percentages in parentheses were obtained from the number of thrombosis types divided by the total number of thrombosis (82/146= 56%), by men (M) or women (W).

(†) The percentages in parentheses were obtained from the number of thrombosis types divided by the total number of spontaneous or non–spontaneous events.

(‡) The percentages in parentheses were obtained from the number of men (M), women (W) or total divided by the total number of patients.

Figure 1. Distribution of aggregation response (in % maximum aggregation (MA)) to 0.5 µM of ADP and 0.5 µM of EPI using PRP by the LTA method.

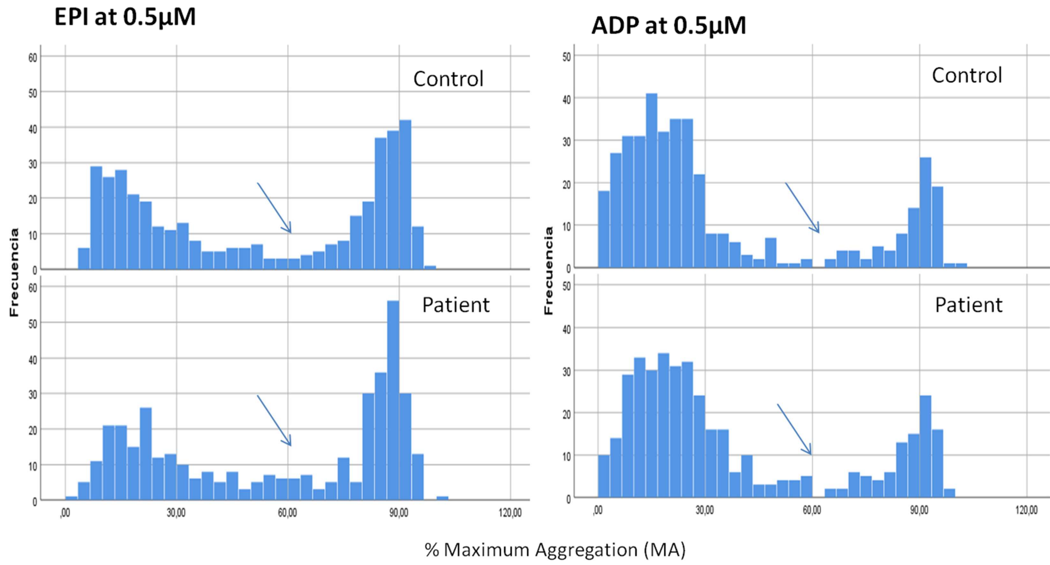


Table 3. Percentile (5 to 95) of % area under the curve of aggregation (AUC) in both agonists (EPI and ADP) in patients and in controls. LTA method with PRP was used. * Statistically significant results ($p<0.05$).

	ADP_AUC		EPI_AUC	
Percentile	Patients	Controls	Patients	Controls
5	1.15*	0.42	6.65*	5.26
10	3.21*	1.68	9.07*	6.95
25	8.18*	5.68	14.35*	11.98
50	19.1*	12.92	27.01*	22.53
75	38.03*	29.59	46.93*	43.98
90	63.08*	62.64	56.32*	54.95
95	69.53*	70.14	60.67	58.29

Figure 2. Distribution of aggregation response (in % area under the curve of aggregation (AUC)) to 0.5 µM of EPI (EPI_AUC) and 0.5 µM of ADP (ADP_AUC) using PRP by LTA method.

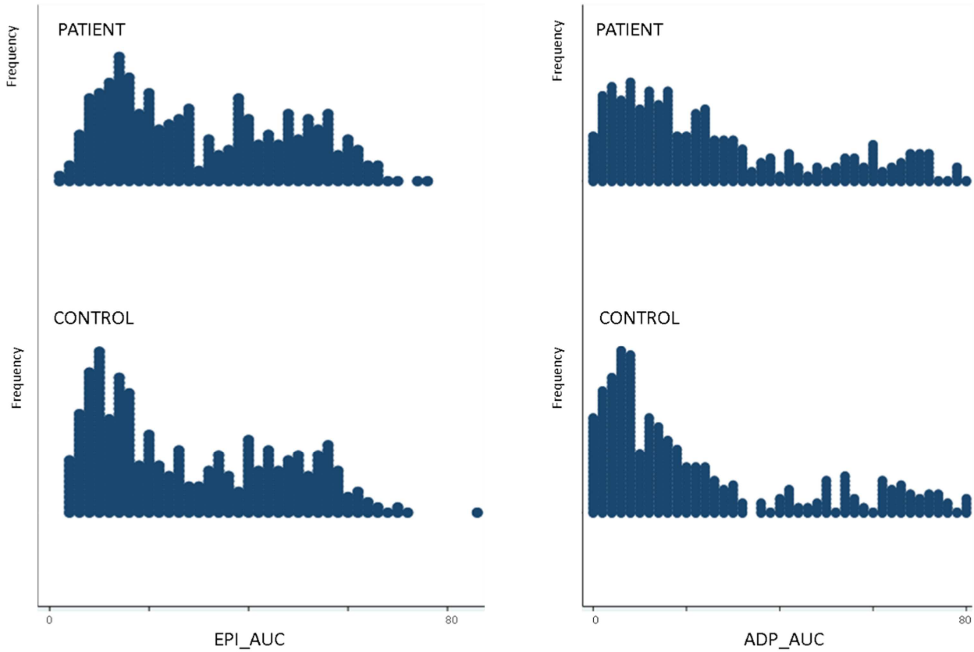


Table 4. The 75th percentile of ADP_AUC (≥29.59) and 50th percentile of EPI_AUC (≥22.53) in patients and controls with PRP using the LTA method for analysis. *Significant different results. Non−significance is listed as ns. ADP_AUC: % area under the curve of aggregation at 0.5 μM of ADP; EPI_AUC: % area under the curve of aggregation at 0.5 μM of EPI.
The *odds ratios* (OR) within the 95% confidence intervals (CI) in parentheses for all of the models: Model 1: non-adjusted model. Model 2: adjusted for sex. Model 3: adjusted for age and sex.

		INDIVIDUAL		
		PATIENTS	CONTROLS	Total
ADP_AUC	<=29.59 (Hypo-ADP)	249 (67.3%)	294 (74.8%)	543
	>29.59 (Hyper-ADP)	121 (32.7%)*	99 (25.2%)*	220
	Total	370	393	763
	Model 1	OR= 1.44 (1.05-1.98 95%IC) (p=0.032)		
	Model 2	OR = 1.38 (1.04-1.84 95%IC) (p=0.025)		
	Model 3	OR=1.14 (0.84-1.55 95% IC) (ns)		
EPI_AUC	<=22.53 (Hypo-EPI)	160 (42.6%)	199 (50.4%)	359
	>22.53 (Hyper-EPI)	216 (57.4%)*	196 (49.6%)*	412
	Total	376	395	771
	Model 1	OR= 1.37 (1.03-1.82) (p=0.031)		
	Model 2	OR=1.47 (1.07-2.02 95% IC) (p=0.037)		
	Model 3	OR=1.17 (0.83-1.64 95% IC) (ns)		

Table 5. Behavior of platelet agggregability with age using PRP by LTA method. Young individuals ranged in age from 18-35 years, Middle ranged from 36-60 and Elderly >60 years. *Statistically significant results. n=number of individuals.

		Hypo-EPI	Hyper-EPI		Hypo-ADP	Hyper-ADP	
		<=22.53	>22.53		<=29.59	>29.59	
Patients	Young (n, (%))	26 (55.3)	21 (44.7)*	p=0.002	38 (84.4)	7 (15.6)*	p=0.001
	Middle (n, (%))	59 (51.8)	55 (48.2)*		84 (74.3)	29 (25.7)*	
	Elderly (n, (%))	75 (34.9)	140 (65.1)*		127 (59.9)	85 (40.1)*	
Controls	Young (n, (%))	81 (60.4)	53 (39.6)*	p=0.014	109 (81.3)	25 (18.7)*	p=0.012
	Middle (n, (%))	76 (46.6)	87 (53.4)*		122 (75.8)	39 (24.2)*	
	Elderly (n, (%))	42 (42.9)	56 (57.1)*		63 (64.3)	35 (35.7)*	

Figure 3. Comparison of fibrinogen levels in hypo- and hyper-responders to platelet aggregability between patients and controls. Platelet aggregation was determined in PRP by LTA method. Total hypo-responders included hypo-ADP and hypo-EPI; Total hyper-responders included hyper-ADP and/or hyper-EPI.

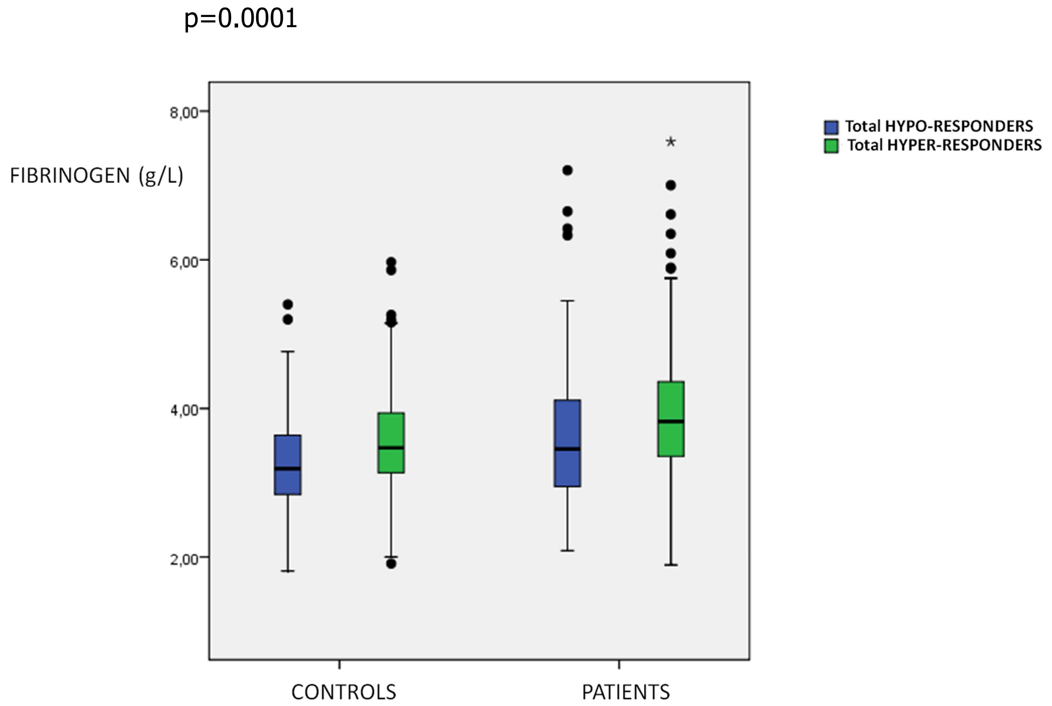


Figure 4. Distribution of aggregation response (in % maximum aggregation (MA)) to 0.5 μM of ADP in controls and in patients tested by Multiplate® analyzer with whole blood impedance aggregometry.

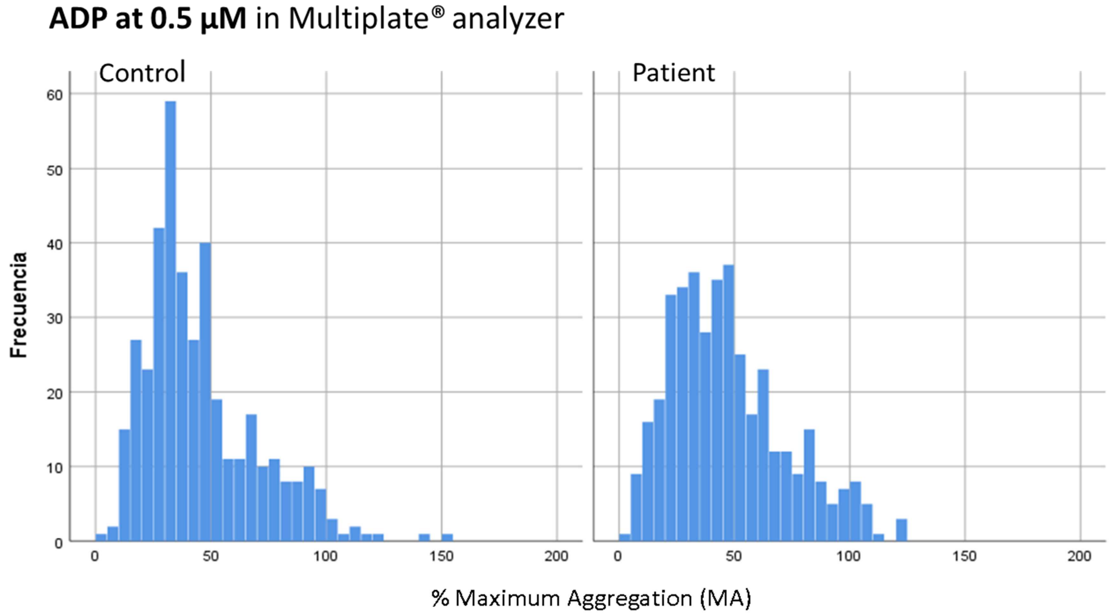


Table 6 Comparison of median aggregation in PRP by LTA method and whole blood impedance aggregometry by Multiplate analyzer®. It was tested aggregation in PRP by LTA method: with ADP (% maximum aggregation (ADP_MA), % area under the curve (ADP_AUC)) and EPI (% maximum aggregation (EPI_MA), % area under the curve (EPI_AUC)). It was tested whole blood impedance aggregometry with ADP by Multiplate® analyzer (% maximum aggregation (Multiplate ADP_MA) and % area under the curve (Multiplate ADP_AUC)). The non−significance are listed as ns.

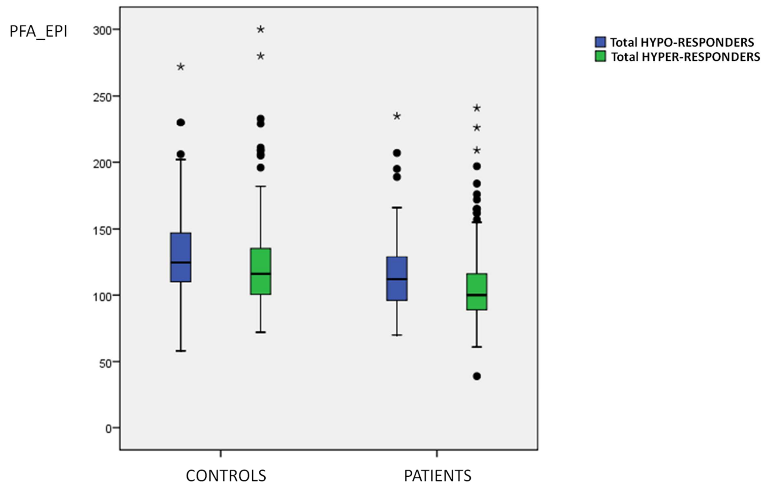
	Median		
	PATIENTS (%)	**CONTROLS (%)**	**p**
ADP_ MA	24.80	22.05	0.022
ADP _AUC	19.10	12.92	**0.001**
EPI_ MA	56.7	52.55	ns
EPI_AUC	27.01	22.53	**0.014**
Multiplate ADP_MA	47.16	45.21	ns
Multiplate ADP_AUC	25.79	24.69	ns

Table 7. Correlations between aggregation in PRP by the LTA method, whole blood impedance aggregometry by Multiplate® analyzer and global platelet function using PFA-100®. It was tested aggregation in PRP by LTA method: EPI_AUC: % Area Under the Curve of EPI aggregation; EPI_AM: % Aggregation Maximum of EPI; ADP_AUC: % Area Under the Curve of ADP aggregation, ADP_AM: % Aggregation Maximum of ADP; It was tested whole blood impedance aggregometry with ADP by Multiplate® analyzer: MULTIPLATE_ADP; It was tested global platelet function using PFA-100®: PFA_EPI: closure times of PFA-100® with EPI as agonist; PFA_ADP: closure times of PFA-100® with ADP as agonist. The non−significance are listed as ns. * Correlation coefficient adjusted for age and sex.

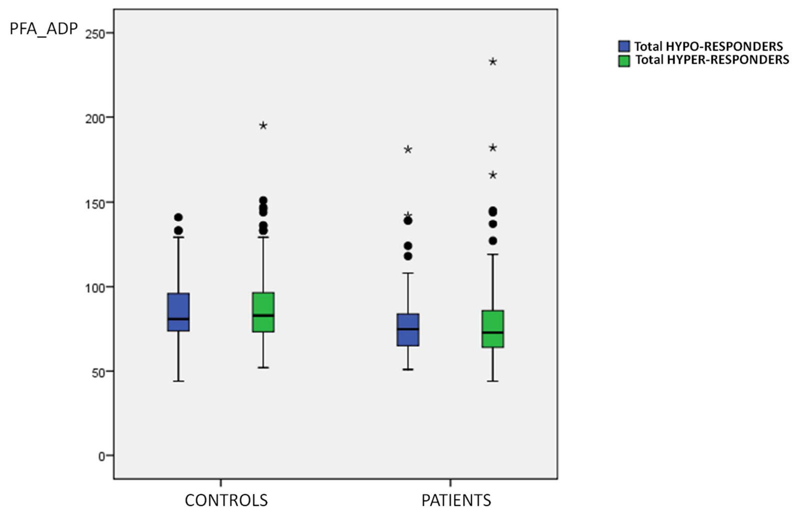
	EPI_AUC	**EPI_AM**	**ADP_AM**	**MULTIPLATE_ADP**	**PFA_EPI**	**PFA_ADP**
ADP_AUC	0.659 p <0.01	0.629 p <0.01	0.954 p <0.01	0.143 p <0.01	**-0.215** **-0.322*** p <0.01	-0.08 (ns)
EPI_AUC	-	0.954 p <0.01	0.653 p <0.01	0.175 p <0.01	**-0.28** **-0.342*** P<0.01	-0.072 (ns)

Figure 5. Comparison of closure times of PFA-100® [a) PFA_EPI and b) PFA_ADP] in hypo- and hyper-responders to platelet aggregability between patients and controls. Platelet aggregation was determined in PRP by the LTA method. Total hypo-responders included hypo-ADP and hypo-EPI; Total hyper-responders included hyper-ADP and/or hyper-EPI.

a) p=0.0001



b) non-significance results



CAPÍTOL 4.2

VAMP8 and SEROTONIN TRANSPORTER LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN A SPANISH FEMALE POPULATION. RESULTS FROM THE RETROVE PROJECT

CAPÍTOL 4.2

VAMP8 and SEROTONIN TRANSPORTER LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN A SPANISH FEMALE POPULATION. RESULTS FROM THE RETROVE PROJECT

RESUM:

Introducció: La hiperreactivitat plaquetària ha estat associada a trombosi i a nivells alts de Proteïna humana de membrana associada a vesícules 8 (VAMP8) i del transportador de la Serotonina (SERT). Dos polimorfismes (*rs1010* del gen de VAMP8 i del gen de *SERT* (*SLC6A4*) estan associats a trombosi arterial.
Objectiu: Determinar si els nivells de serotonina, SERT i/o VAMP8 i aquests polimorfismes estan associats al risc de trombosi venosa.
Material i mètodes: Un total de 324 individus es van incloure del projecte RETROVE (Riesgo de Enfermedad TROmboembólica Venosa).
VAMP8, SERT i serotonina es van determinar per ELISA; el polimorfisme de *SLC6A4* i VAMP8 per reacció en cadena de la polimerasa (PCR) i PCR a temps real. El risc de trombosi venosa es va calcular mitjançant regressió logística per a estimar la OR crua i ajustada per sexe, edat, índex de massa corporal i cofactors de risc de trombosi venosa.
Resultats: Es van trobar nivells alts de VAMP8 i SERT estadísticament significatius en els pacients respecte als controls. Per contra, la serotonina tenia nivells més baixos en els pacients que en els controls. Quan els individus es van estudiar segons el gènere, només les dones van exhibir diferències estadísticament significatives. La OR pel VAMP8 va ser de 3,25 (1,61-6,56 IC 95%). La OR ajustada no va canviar. La OR del SERT va ser de 2,76 (1,36-5,60 IC 95%), la OR ajustada també es va mantenir. Per la serotonina, amb una OR de 2.62 (1,40-4,92 IC 95%), la OR ajustada no era significativa. Per contra els homes no van mostrar diferències significatives.
No es van trobar diferències estadísticament significatives entre pacients i controls pels dos polimorfismes.
Conclusions: Els nivells de VAMP8 i SERT s'associen a trombosi venosa en població espanyola femenina.

Thrombosis Research 181 (2019) 99–105

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

VAMP8 and serotonin transporter levels are associated with venous thrombosis risk in a Spanish female population. Results from the RETROVE Project

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ARTICLE INFO

Keywords:
VAMP8
SERT
Serotonin
V-SNARE
Platelet granules
Platelet hyper-reactivity
Thrombosis

ABSTRACT

Introduction: Platelet hyper-reactivity has been associated with thrombosis and high levels of human vesicle-associated membrane protein 8 (VAMP8) and serotonin transporter (SERT). Two polymorphisms (*rs1010* of *VAMP8* gene and in *SERT* gene (*SLC6A4*)) are associated with arterial thrombosis.
Aim: To determine if levels of serotonin, SERT and/or VAMP8 and these polymorphisms are associated with the risk of venous thrombosis.
Material and methods: A total of 324 individuals were included in the RETROVE Study (Riesgo de Enfermedad TROmboembólica VEnosa).

VAMP8, SERT and serotonin were determined by ELISA; polymorphisms of *SLC6A4* and VAMP8 by polymerase chain reaction (PCR) and real time PCR. The venous thrombotic risk was calculated by a logistic regression method to estimate the crude and adjusted OR (adjusted for sex, age, body mass index and venous thrombosis risk co-factors).
Results: Statistically significant high levels of VAMP8 and SERT were found in patients, but not in controls. In contrast, serotonin showed lower levels in patients than in controls. When individuals were studied by gender, only women exhibited a statistically significant difference: the OR for VAMP8 was 3.25 (1.61–6.56 95% CI). The adjusted OR did not change. The OR for SERT was 2.76 (1.36–5.60 95% CI), the adjusted OR was maintained also. For serotonin with OR of 2.62 (1.40–4.92 95% CI), the adjusted OR was not significant. In contrast males did not show significant differences.

No statistically differences between patients and controls were found for both polymorphisms.
Conclusions: VAMP8 and SERT levels are associated with venous thrombosis in a female Spanish population.

1. Introduction

Platelet hyper-reactivity has been associated with venous [1] and arterial [2–5] thrombosis. The hyper-reactive platelet phenotype is characterized by an increase in vitro platelet aggregation after activation with low concentrations of adenosine diphosphate (ADP) and/or epinephrine (EPI). There is a bimodal distribution with hypo-reactive individuals (aggregation < 40%) and hyper-reactive individuals (aggregation > 60%) [6,7].

When platelet aggregation is induced by epinephrine, there is a release of cargo that is contained in platelet granules. The interactions of membrane proteins in platelet plasma membrane (t-SNARE) and in the granules (v-SNARE) form a heteromeric complex. This extends to the two bilayers and mediates membrane fusion and granule cargo release [8].

Human vesicle-associated membrane protein 8 (VAMP-8) is a v-SNARE. It is the first SNARE implicated in the secretion of platelet granules as it was demonstrated by analysis of platelets from knockout mice in VAMP8 [9]. High levels of VAMP8 are found in the hyper-reactive platelet phenotype [10].

Also, the Cardiovascular Health Study [11] reported that there is a strong association between the VAMP8 *rs 1010* single nucleotide

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https://doi.org/10.1016/j.thromres.2019.07.023
Received 4 April 2019; Received in revised form 19 July 2019; Accepted 25 July 2019
Available online 26 July 2019
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Table 1
Clinical characteristics of patients and controls in whom VAMP8, SERT and serotonin values were determined.

Baseline characteristics[a]	Patients		Controls		p[†]
	Males	Females	Males	Females	
Gender (n)	66	79	78	101	
Age (year) (mean ± SD)	61.4 ± 17.3	64.6 ± 19.5	44.6 ± 17.3	48.0 ± 19.4	0,0001
BMI (kg·m^{-2}) (mean ± SD)	27.5 ± 3.9	27.6 ± 4.9	25.5 ± 3.6	25.0 ± 4.2	0,0001
Smoking (n, %)	9 (13.6)	5 (6.3)	17 (21.8)	15 (14.8)	NS
Alcohol consumption (n, %)	35 (53.0)	33 (41.7)	55 (70.5)	45 (44.5)	NS
Hypertension (n, %)	30 (45.4)	36 (45.5)	9 (11.5)	19 (18.8)	0,0001
Dyslipidaemia (n, %)	22 (33.3)	27 (34.2)	12 (15.4)	22 (21.7)	NS
Statins (n, %)	17 (25.7)	20 (25.3)	7 (8.9)	16 (15.8)	NS
Diabetes mellitus (n, %)	8 (12.1)	7 (8.8)	3 (3.8)	4 (3.9)	NS
Autoimmune disease (n, %)	6 (9,09)	10 (12.6)	5 (6.4)	9 (8.9)	NS
Arterial thrombosis background (n, %)	5 (7.6)	6 (7.6)	–	1 (1.0)	0.002
Oral Contraceptives	–	32 (41)	–	55 (54.5)	NS
Non–steroidal anti–inflammatory drugs (n, %)	9 (13.6)	21 (26.6)	14 (17.9)	19 (18.8)	NS
Anti–platelet drugs (n, %)	8 (12.1)	8 (10.1)	1 (1.3)	2 (1.9)	0,023

Non-significance is listed as NS.
[a] Number of individuals in each group. The percentages are given in parentheses.
[†] The statistical differences, fixed in a $p < 0.05$, are reported only for descriptive purposes.

polymorphism and the risk of myocardial infarction. This polymorphism is associated also with hyper-reactive platelet phenotypes [10].

It seems to be a "global phenomenon", so subjects with increased platelet aggregation in response to one inductor (as EPI), show an increase response also to other platelet inductors. Aggregation was inducted in a high percentage of individuals when serotonin (5-TH) was added to sub-maximal concentration of EPI, than when EPI alone was added. Also, the bimodal distribution was retained [7]. The combination of serotonin and ADP caused irreversible aggregation of platelets.

Serotonin has been considered a weak agonist for human platelets, but it enhances platelet activation and pro-coagulant responses. Also, it augments thrombogenesis of injured vascular surfaces [12]. Then, serotonin seems to have a significant agonistic effect on platelets.

There is a receptor for serotonin (5-HT$_{2A}$) on the platelet surface and a serotonin transporter (SERT) that re-uptakes serotonin. SERT moves serotonin into platelet-dense bodies [13], and is secreted during platelet activation. Hyper-reactive platelet individuals exhibited increased SERT levels and increased binding affinity of serotonin-SERT [7]. SERT is blocked by selective inhibition of serotonin re-uptake (SSRI). Then, the effects of serotonin on platelet activation are weakened and down-regulated the pro-thrombotic tendencies [12]. SERT is present in platelet and in neurons.

A case-control study [14] reported that SSRI is associated with protection against myocardial infarction. Notably, there is a polymorphism in the promoter region of *SERT* gene (*SLC6A4*). It is located around 1 kb from the transcription initiation site and consists of a 44-bp deletion or insertion. The long variant (LL) has more transcriptional activity than the short variant (SS).

The Cas-Témoins de l'Infarctus du Myocarde (ECTIM) Study [15] reported that there is an association with the *LL* genotype of the *SLC6A4* polymorphism and a higher risk of myocardial infarction.

The aim of our study was to determine if VAMP8, SERT and serotonin levels are associated with the risk of venous thrombosis. In addition, we determined if the *rs 1010* SNP of VAMP8 and a polymorphism in the promoter region of the *SLC6A4* are associated with this risk.

2. Materials and methods

2.1. Study design

Patients and controls were recruited from the RETROVE Study (Riesgo de Enfermedad TROmboembólica VEnosa) between 2012 and 2016 in our Thrombosis and Haemostasis Unit (Hospital de la Santa Creu i Sant Pau, Barcelona (Spain)). RETROVE is a prospective, observational, case-control study that consisted of 400 consecutive patients with venous thrombosis and 400 healthy controls. Detailed population baseline characteristics were described previously [16]. The goal of the RETROVE study was to obtain a mathematical algorithm that defines the individual risk of suffering a venous thrombotic event. Controls were not matched intentionally for sex or for age. Thus, any statistical inference was adjusted for these co-variates.

Patients 18 years old were included in our study. They had at least one venous thrombotic event diagnosed with doppler ultrasonography, tomography, magnetic resonance, arteriography, phlebography and pulmonary gammagraphy. As detailed previously [16], a venous thrombotic event was classified as either spontaneous or unprovoked or non–spontaneous or provoked. The provoking factors (one or more) were within three months previous to an event [17,18]. Provoking factors were: surgery, pregnancy or puerperium, immobilization, oral contraceptives, prothrombotic non–neoplastic diseases and other circumstances.

The control individuals were not blood donors and they were not related to the patients. They were distributed according to age and sex in a Spanish population (2001 census).

All of our procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau in Barcelona, Spain. Written informed consent (in accordance with the Declaration of Helsinki) was obtained from all participants.

We began our study after the RETROVE Project had started, so we were able to obtain Platelet-rich plasma (PRP) for measurement of VAMP8, SERT and serotonin in 324 individuals: 145 patients with venous thrombosis (66 men and 79 women) and 179 healthy controls (78 men and 101 women) selected chronologically in the same previous conditions of RETROVE project but in a shorter period of time (between November 2013 to May 2015). Table 1 lists the clinical characteristics of this population. We found that age, body mass index and hypertension had statistical differences. These parameters also had statistical differences in the total RETROVE population [16] consisting of 400 consecutive patients with venous thrombosis and 400 healthy controls.

Table 2 shows the venous thrombosis events that were classified as either unprovoked or spontaneous or provoked or non–spontaneous.

2.2. Blood collection

Blood was collected 6 months after the most recent thrombotic event. Anti-vitamin K was withdrawn and the blood samples were taken

Table 2
Characteristics of 145 consecutives thrombotic events where VAMP8, SERT and serotonin levels were determined.

	Spontaneous			Non-spontaneous		
	M[a]	F[a]	Total[b]	M[a]	F[a]	Total[b]
Isolated deep vein thrombosis	25 (52.0)	21 (44.7)	46 (48.4)	10 (55.5)	11 (34.3)	22 (44.0)
No isolated deep vein thrombosis	8 (16.6)	5 (10.6)	13 (13.7)	3 (16.6)	6 (18.7)	9 (18.0)
Isolated pulmonary embolism	13 (27.0)	19 (40.4)	32(33.6)	5 (27.7)	14 (43.7)	19 (38.0)
Visceral thrombosis	1 (2.0)	–	1 (1,0)	–	–	–
Venous sinus thrombosis	1 (2.0)	2 (4.2)	3 (3.1)	–	1 (3.1)	1 (2.0)
Total[c]	48 (33.1)	47 (32.4)	95(65.5)	18 (12.4)	32 (22.1)	50 (34.5)

[a] The percentages between parentheses were obtained from the number of thrombosis types divided by the total number of males (M) or females (F).
[b] The percentages between parentheses were obtained from the number of thrombosis types divided by the total number of spontaneous or non–spontaneous events.
[c] The percentages between parentheses were obtained from the number of male (M), female (F) or total divided by the total number of patients.

after a washout period of at least 21 days or 36 h for heparin. Also, we required a week for anti-platelet therapy (aspirin or clopidogrel) or for other drugs that affect platelet function such as non-steroidal anti-inflammatory (NSAID). For SERT and serotonin analysis, 4 patients and 2 controls were excluded because they were being treated with serotonin reuptake inhibitor (SSRI). Blood samples were collected from the antecubital vein and immediately anticoagulated with 1/10 volume of 0.129 M sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 160*g* for 10 min. Platelet-poor plasma (PPP) was obtained by centrifugation at 2000*g* for 20 min. It was frozen and stored at − 40 °C until analyzed.

2.3. Laboratory determinations

VAMP 8 was determined with human vesicle-associated membrane protein 8 (VAMP8) ELISA kit by CUSABIO (Houston, USA). Platelets were isolated: PRP was washed with an equal volume using a phosphate buffer solution (Na_2HPO_4-KH_2PO_4) and centrifugation at 4500*g* for 10 min. The platelet pellet was frozen at − 40 °C. Before analysis, the platelet pellet was resuspended with Triton X-100 and cooled on ice for 1 h. The final concentration was expressed as the number of platelets (pg/10^9 PLT).

SERT was determined by a commercial kit: ELISA for serotonin transporter (SERT) by USCN Life Science Inc. (Wuhan, Hubei) according to the manufacturer's instructions. Platelets were isolated as described above. The final concentration was expressed as the number of platelets (pg/10^9 PLT).

Serotonin was determined by a serotonin ELISA kit (IBL international GMBH, Hamburg, Germany) according to the manufacturer's instructions. Platelets were isolated as described above. The platelet pellet was resuspended with 200 µL of distilled water. The final concentration was expressed as the number of platelets (ng/10^9 PLT).

2.4. Genetic analysis

DNA was isolated from peripheral blood leukocytes by a standard protocol [19].

The **VAMP8 *rs 1010* single nucleotide polymorphism**: Genotyping for *rs1010* was performed using the *TaqMan* SNP Genotyping by allelic assay (ID C_2091644_20; Applied Biosystems, Foster City, CA, USA). Fluorescence data were obtained using an ABI 7500 Real Time PCR System (Applied Biosystem).

The **polymorphism of the *SLC6A4* promoter** was analyzed by direct PCR as previously described [20] with minor modifications. The final amplicon product consisted of 406/450-bp fragments (*S* and *L* alleles, respectively). PCR was performed with Master Mix Promega (Madison, USA).

2.5. Statistics analyses

The controls and patients were compared by the Mann-Whitney test. Interquartiles (25th and 75th percentiles) and median were calculated. 75th percentile was selected as the cut off (≥ 1930 pg/10^9 PLT) of VAMP8 levels and (≥ 784 pg/10^9 PLT) of SERT levels. A 25th percentile was selected as cut off (≤ 115 ng/10^9 PLT) of serotonin levels. The interaction between these biomolecules was analyzed for age and sex by an ANOVA. The $\chi 2$ test was used for group comparisons of frequencies. A logistic regression method was used to estimate both the crude and adjusted odds ratio (OR) of venous thrombosis risk with 95% confidence intervals. A report of previous studies [21,22] stated that potential confounders can add partiality in the determination of the risk factors for thrombosis. With venous thrombosis, age, sex, body mass index (BMI), Factor V Leiden (FVL) and high levels of Factor VIII (FVIII) ((≥ 217%) and Factor von Willebrand (FvW) (≥ 183%) were potential confounders. To avoid confusion, we calculated the venous thrombosis risk applying three statistical models. The venous thrombosis risk models were for VAMP8: 1) unadjusted or crude (model 1); 2) adjusted for age (model 2); 3) adjusted for age, BMI, FVL, FVIII and FvW; for SERT and serotonin: 1) unadjusted or crude (model 1); 2) adjusted for BMI (model 2); 3) adjusted for age and BMI (model 3). Finally, p values < 0.05 were considered statistically significant.

3. Results

We found differences for categorical variables as shown in Table 1. Also, we observed 145 consecutive events of thrombosis. There was more spontaneous (n = 95, 65.5%) than non-spontaneous venous thrombosis (n = 50, 34.5%) (Table 2).

It is notable that VAMP8 and SERT showed statistically significant higher levels in patients than in controls. In contrast, serotonin showed lower levels in patients than in controls as is shown in Table 3.

We found that the function biomolecules were affected by sex.

Our analysis of VAMP8 showed a clear interaction with sex (p = 0.028). The control values of women were lower than the control values of men and patients (Fig. 1a).

Table 3
Levels of VAMP8, SERT and serotonin in patients and in controls.

	Median		25th percentile		75th percentile		p
	Patient	Control	Patient	Control	Patient	Control	
VAMP8 (pg/10^9 PLT)	1498	1138	903	536	2372	1930	0,001
SERT (pg/10^9 PLT)	636	543	454	366	989	784	0,001
Serotonin (ng/10^9 PLT)	144	172	69	115	206	263	0,013

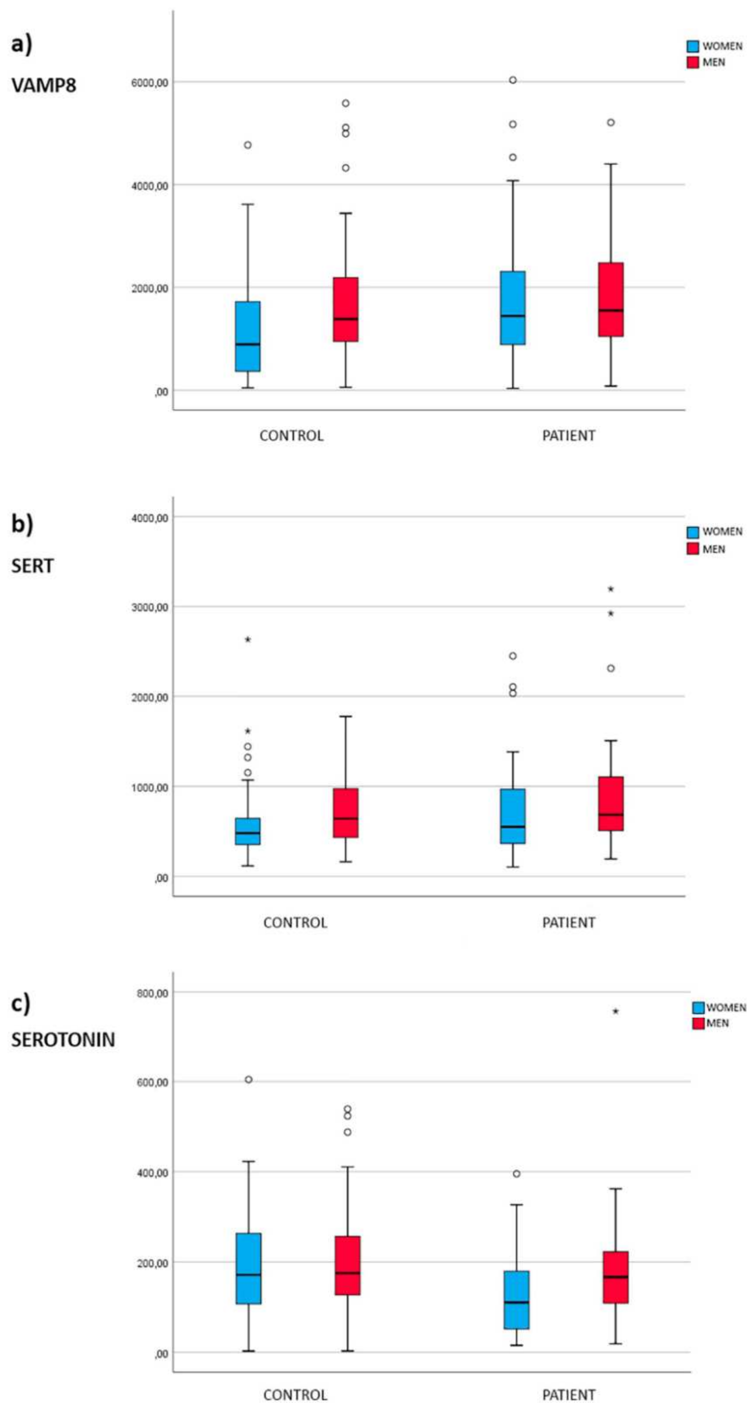


Fig. 1. Levels of VAMP 8 (a), SERT (b), and serotonin (c) by sex.

Table 4
75th percentile of VAMP8 (≥1930 pg/10^9 PLT) and SERT (≥784 pg/10^9 PLT) and 25th percentile of serotonin (≤115 ng/10^9 PLT) in women and men.

		Women		Men	
		Patient	Control	Patient	Control
VAMP8	< 1930 (pg/10^9 PLT)	49 (62%)	85 (84.2%)	45 (68.2%)	50 (64.1%)
	≥1930 (pg/10^9 PLT)	30 (38%)[a]	16 (15.8%)[a]	21 (31.8%)	28 (35.9%)
	Model 1 OR (95% CI)	3,25 (1.61–6,56) p = 0,001		0,83 (0,41–1,67) NS	
	Model 2 OR (95% CI)	3,07 (1,43–6,59)p = 0,004		0,84 (0,39–1,83) NS	
	Model 3 OR (95% CI)	3,43 (1,41–8,35) p = 0,006		0,59 (0,23–1,49) NS	
SERT	< 784 (pg/10^9 PLT)	51 (66.2%)	84 (84.8%)	37 (57.8%)	49 (62.8%)
	≥784 (pg/10^9 PLT)	26 (33.8%)[a]	15 (15.2%)[a]	27 (42.2%)	29 (37.2%)
	Model 1 OR (95% CI)	2.76 (1.36–5.6) p = 0,004		1.24 (0.63–2.42) NS	
	Model 2 OR (95% CI)	2.39 (1.14–4.99) p = 0,021		1.35 (0.67–2.73) NS	
	Model 3 OR (95% CI)	2.25 (1.08–4.70) p = 0,030		0.98 (0.46–2.12) NS	
Serotonin	≤115 (ng/10^9 PLT)	40 (51,9%)[a]	28 (29.2%)[a]	18 (29.0%)	15 (19.7%)
	> 115 (ng/10^9 PLT)	37 (48.1%)	68 (70.1%)	44 (71.0%)	62 (80.3%)
	Model 1 OR (95% CI)	2.62 (1.40–4.92) p = 0,003		1.69 (0.77–3.71) NS	
	Model 2 OR (95% CI)	2.17 (1.13–4.17) p = 0,020		1,48 (0.65–3.36) NS	
	Model 3 OR (95% CI)	1.67 (0.85–3.29) NS		1.62 (0.72–3.66) NS	

Non-significance is listed as NS.
The *odds ratios* (OR) within the 95% confidence intervals (CI) in parentheses for all of the models. By VAMP8: Model 1: non-adjusted model. Model 2: adjusted for age. Model 3: adjusted for age, BMI, FVL, FVIII and FvW.
By SERT and serotonin: Model 1: non-adjusted model. Model 2: adjusted for BMI. Model 3: adjusted for age and BMI.
[a] Significant different results.

Table 5
Non-spontaneous and spontaneous venous thrombosis in patients and controls in the 75th percentile of VAMP8 (≥1930 pg/10^9 PLT). Non-significance is listed as NS.

		VAMP8 percentile 75th			
		< 1930 (pg/10^9 PLT)	≥1930 (pg/10^9 PLT)	Total	
Women	Controls	85 84,20%	16 15,80%	101 100,00%	
	Patients with **non-spontaneous** thrombotic event	22 68,80%	10 31,3%*	32 100,00%	
	Patients with **spontaneous** thrombotic event	27 57,40%	20 42,6%*	47 100,00%	**p = 0,002**
	Total	134 74,40%	46 25,60%	180 100,00%	
Men	Controls	50 64,10%	28 35,90%	78 100,00%	
	Patients with **non-spontaneous** thrombotic event	13 72,20%	5 27,80%	18 100,00%	
	Patients with **spontaneous** thrombotic event	32 66,70%	16 33,30%	48 100,00%	**NS**
	Total	95 66,00%	49 34,00%	144 100,00%	

*Significant different results.
Relevant.

We found that SERT levels were sex-depended (p = 0.0001). It is clear that women have lower levels of SERT than men both in patients and in controls (Fig. 1b).

Sex affects serotonin levels also (p = 0.011). The values for women were lower than for men in patients and in controls (Fig. 1c).

The levels of VAMP8 and SERT were studied in individuals who were in the 75th percentile (≥1930 pg/10^9 PLT by VAMP8 levels and ≥784 pg/10^9 PLT by SERT levels): A higher percentage of patients had levels in the upper percentile 75th as by VAMP8 as SERT levels (35.2% vs 24.6%; difference 10.6%, p = 0.049 and 40.2% vs 24.9%; difference 15.3%, p = 0.002 respectively).

3.1. VAMP8

When gender was included in our study of VAMP8 levels, we noted a significant difference but only in women: 38.0% vs 15.8%; difference of 22.2%; p = 0.001. The associated thrombotic OR was 3.25 (1.61–6.56 95% CI). The OR did not change when co-variates were included (OR = 3.43 (1.41–8.35 95% CI) p = 0.006) (Table 4).

3.2. SERT

As noted previously, when gender was included, women exhibited significant differences: 33.8% vs 15.2%; difference of 18.6%; p = 0.004. The associated thrombotic OR was 2.76 (1.36–5.60 95% CI). When the co-variates were included, the OR was maintained (OR = 2.25 (1.08–4.70) (p = 0.030)) (Table 4).

Importantly, males did not show significant differences between patients and controls with VAMP8 or with SERT as shown in Table 4.

We studied the effect of serotonin in individuals in the 25th percentile (≤115 ng/10^9PLT):

3.3. Serotonin

Serotonin behaved opposite to SERT and VAMP8. It showed a higher percentage of patients with levels under the 25th percentile (40.8% vs 26.3%; difference 14.5% p = 0.006). When individuals were studied by sex, a significant difference was obtained in women (51.9% vs 29.2%; difference 22.7%; p = 0.004). The associated thrombotic OR was 2.62 (1.40–4.92 95% CI). When age was included, the significant difference was lost (OR = 1.67 (0.85–3.29 95% CI). Males did not show differences between patients and controls (Table 4).

We found an association in the 75th percentile of VAMP8 levels and spontaneous venous thrombosis also in women (42.6% vs 31.3% difference 11.3% p = 0.002) as shown in Table 5.

***The VAMP8 rs 1010* single nucleotide polymorphism and *SLC6A4* polymorphism**: No statistical differences between patients and controls were found as is show in Table 6.

4. Discussion

One important result of our study is that in a Spanish population, levels of VAMP8 and SERT are associated with venous thrombotic risk,

Table 6
Allelic frequencies of VAMP8 *rs1010 SNP* and SERT genotype (polymorphism in the promoter region of the *SLC6A4*). Non-significance is listed as NS.

VAMP (genotype)	AA	AG	GG	p
Patient (%)	34	48	18	NS
Control (%)	36	42	22	NS
SERT (genotype)	LL	LS	SS	p
Patient (%)	33	45	22	NS
Control (%)	27	47	26	NS

but only in women. By controlling the potential confusion, the thrombotic risk factor did not decrease after adjusting for age. In our study the controls were not matched with patients for age and sex due to our study design. Our controls were younger than the patients. Nevertheless, our results were clear-cut. As far as we know, there are no other studies that examined this association.

High levels of VAMP8 and SERT are associated with platelet hyperreactivity. Interestingly this phenotype has been associated with females [6]. Berlin et al. [23] found a higher platelet aggregation in women of fertile age than in post-menopausal women and men. This could be a mechanism to prevent heavy menstrual bleedings. Also, we found higher levels in women of fertile age than in menopausal women (results not shown).

To account for the relationship between stress and thrombotic events, it has been proposed that a synergistic interaction occurs between adrenergic and serotonergic stimuli [24,25]. These thrombotic events include myocardial infarction and sudden death. Different types of receptors control the actions of serotonin that are ended by a single serotonin transporter (SERT). So, SERT is the main mechanism that regulates plasma serotonin levels. It prevents vasoconstriction and thus ensures a stable blood flow [26]. In our study, we found that low levels of Serotonin were associated with high venous thrombotic risk, but only in women. High levels of SERT might explain the decrease in the levels of serotonin that we observed.

Platelet pathology has been associated classically with arterial thrombosis. We analyzed the association of VAMP8 and SERT with venous thrombosis adjusted by arterial risk co-factors as body mass index (BMI) and as well, venous risk cofactors as FVIII and FvW high levels.

Our group has reported recently [16] that short times to occlusion of PFA-100 (as EPI as ADP) are associated with venous thrombosis and suggested that these mechanism could be associated with platelet adhesion. We did not find a correlation with VAMP8, SERT or serotonin levels and short values of PFA (both in ADP and in EPI) (data not shown). These biomolecules are related to platelet aggregation not in platelet adhesion so, our results support the hypothesis that the mechanism that is related to short values of PFA-100 with thrombosis could cause platelet adhesion.

An important finding of our study is that there is an association between VAMP8 levels and spontaneous venous thrombosis in women. We analyzed a SNP of VAMP8 that it is associated with high levels of VAMP8 and myocardial infarction: the *rs 1010* SNP in *VAMP8*. It has been observed in five independent patient populations [11,27,28]. We did not find an association between this SNP and venous thrombosis. We analyzed other polymorphism in the *SERT* gene (polymorphism in the promoter region of the *SLC6A4*). This polymorphism is associated [15] with myocardial infarction risk, but we did not find this association with venous thrombosis. Often, polymorphisms associated with arterial thrombosis have not been found associated with venous thrombosis.

There are some limitations in our study. First, because we are a reference center, it is possible that a sample with random group of patients could have lower thrombotic risk than our sample. Second, all biomolecules were determined at least 6 months after the thrombotic event. Thus, if these phenotypes were studied during the acute phase, our results might have been different. Third, there could be a possible selection bias; patients and controls were selected chronologically between November 2013 and May 2015. Our population was smaller (324 individuals) than the total RETROVE population (800 individuals) but the statistical differences found in some of the baseline characteristics between patients and controls (age, body mass index and hypertension) were also found statistical different in the total RETROVE population [16]. Fourth, as it is a case-control study, it is not possible to demonstrate causality; therefore, we demonstrate the association between these biomolecules and the thrombotic risk. Finally, the number of individuals in our study may not provide enough statistical power. Further studies with a larger population may determine the validity of our findings.

In conclusion, our results showed that VAMP8 and SERT levels are related to venous thrombotic risk in women. Although there are two polymorphisms in the gene of these proteins associate with their levels and myocardial infarction, we did not find an association of their allelic frequencies and venous thrombotic risk. Further studies are necessary to confirm our results.

Acknowledgements

We would like to thank Professor William Stone and Dr. Montserrat Borrell for reviewing the manuscript.

Sources of funding

Spanish grants: FIS PI12/00612 and FIS PI 15/0269.

Declaration of competing interest

The authors report no conflicts of interest.

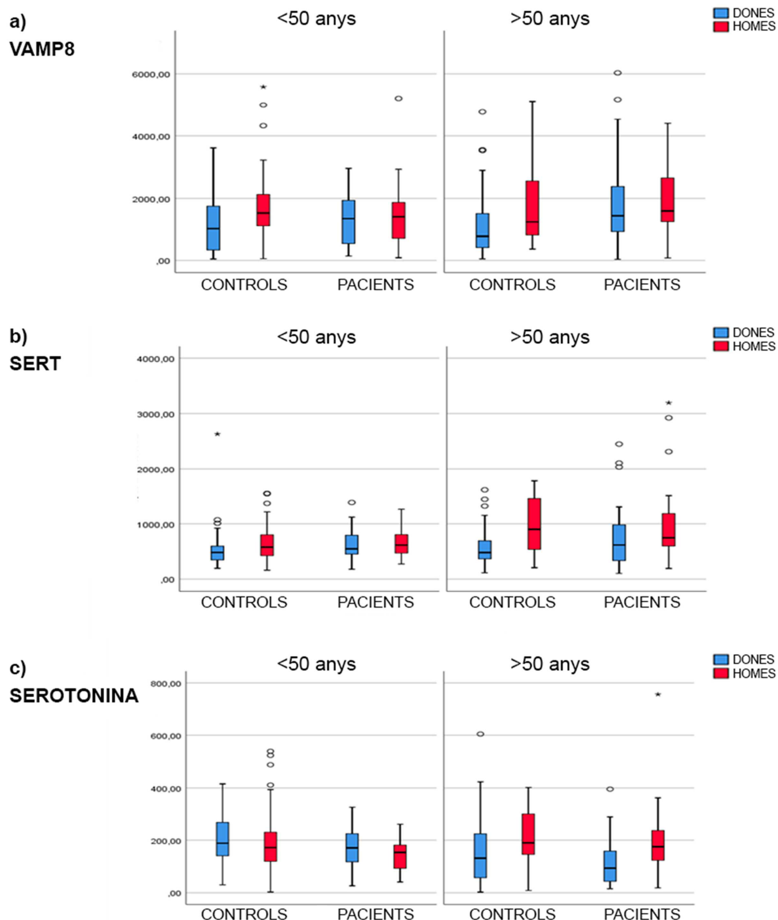
References

[1] C. Hayes, S. Kitahara, O. Tcherniantchouk, Decreased threshold of aggregation to low-dose epinephrine is evidence of platelet hyperaggregability in patients with thrombosis, Hematol Rep 6 (2002) 325–328.
[2] M.D. Trip, V.M. Cats, F.J. van Capelle, J. Vreeken, Platelet hyperreactivity and prognosis in survivors of myocardial infarction, N Engl J Med 322 (1991) 1549–1554.
[3] S.S. Kabbani, M.W. Walkins, T. Ashikaga, et al., Platelet reactivity characterized prospectively: a determinant of outcome 90 days after percutaneous coronary intervention, Circulation 104 (2001) 181–186.
[4] K. Konstantopoulos, J.C. Grotta, C. Sills, K.K. Wu, J.D. Hellums, Shear-induced platelet aggregation in normal subjects and stroke patients, Thromb Haemost 74 (1995) 1329–1334.
[5] K. Vanschoonbeek, M.A. Fejige, J.F. Keuren, et al., Thrombin-induced hyperactivity of platelets of young stroke patients: involvement of thrombin receptors in the subject-dependent variability in Ca2+ signal generation, Thromb Haemost 88 (2002) 931–937.
[6] . L.Yee D, Sun C.W., Bergeron A.L., Dong J, Bray P.F. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 2005; 106: 2723–2729.
[7] J.S. Berger, R.C. Becker, C. Kuhn, M.J. Helms, T.L. Ortel, R. Williams, Hyperrreactive platelet phenotypes: relationship to altered serotonin transporter number, transport kinetics and intrinsic response to adrenergic co-stimulation, Thromb Haemost 109 (2013) 85–92.
[8] Q. Ren, S. Ye, S.W. Whiteheart, The platelet release reaction: just when you thought platelet secretion was simple, Curr Opin Hematol 106 (2008) 2723–2729.
[9] Q. Ren, H.K. Barber, G.L. Crawford, Z.A. Karim, C. Zhao, W. Choi, C.C. Wanf, W. Hong, S.W. Whiteheart, Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction, Mol Biol Cell 18 (2007) 24–33.
[10] A.A. Kondkar, M.S. Bray, S.M. Leal, S. Nagalla, D.J. Liu, Y. Jin, J.F. Dong, Q. Ren, S.W. Whiteheart, C. Shaw, P.F. Bray, VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA, J Thromb Haemost 8 (2) (2010) 369–378.
[11] D. Shiffman, E.S. O'Meara, L.A. Bare, C.M. Rowland, J.Z. Louie, A.R. Arellano, T. Lumley, K. Rice, O. Iakoubova, M.M. Luke, B.A. Young, M.J. Malloy, J.P. Kane, S.G. Ellis, R.P. Tracy, J.J. Devlin, B.M. Psaty, Association of gene variants with incident myocardial infarction in the Cardiovascular Health Study, Arterioscler Thromb Vasc Biol 28 (1) (2008) 173–179.

[12] A.M. Galan, I. Lopez-Vilchez, M. Diaz-Ricart, F. Navalon, E. Gomez, C. Gasto, G. Escolar, Serotonergic mechanisms enhance platelet-mediated thrombogenicity, Thromb Haemost 102 (2009) 511–519.
[13] J.G. White, Platelet secretory granules and associated proteins, Lab Invest 68 (1993) 497–498.
[14] W.H. Sauer, J.A. Berlin, S.E. Kimmel, Selective serotonin reuptake inhibitors and myocardial infarction, Circulation 104 (2001) 1894–1898.
[15] F. Fumeron, D. Betoulle, V. Nicaud, A. Evans, F. Kee, J.B. Ruidavets, D. Arveiler, G. Luc, F. Cambien, Serotonin transporter gene polymorphism and myocardial infarction, Circulation 105 (2002) 2943–2945.
[16] M. Vázquez-Santiago, N. Vilalta, B. Cuevas, J. Murillo, D. Llobet, R. Macho, N. Pujol-Moix, M. Carrasco, J. Mateo, J. Fontcuberta, J.M. Soria, J.C. Souto, Short closure time values in PFA-100 are related to venous thrombotic risk. Results from the RETROVE study, Thromb Research 169 (2018) 57–63.
[17] C. Martinez, A.T. Cohen, L. Bamber, S. Rietbrock, Epidemiology of first and recurrent venous thromboembolism: a population-based cohort study in patients without active cancer, Thromb. Haemost 112 (2014) 255–263, https://doi.org/10.1160/TH13-09-0793.
[18] C. Kearon, W. Ageno, S.C. Cannegieter, B. Cosmi, G.-J. Geersing, P.A. Kyrle, P. and D.V. in T.D. Subcommittees on Control of Anticoagulation, Categorization of patients as having provoked or unprovoked venous thromboembolism: guidance from the SSC of ISTH., J, Thromb Haemost 14 (2016) 1480–1483, https://doi.org/10.1111/jth.13336.
[19] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acid Res. 16 (1988) 1215.
[20] E. Cook Jr., R. Courchesne, C. Lord, N.J. Cox, S. Yan, A. Lincoln, R. Haas, E. Courchesne, B.L. Leventhal, Evidence of linkage between the serotonin transporter and acustic disorder, Molecular Psychiatry 2 (1997) 247–250.
[21] P. Prandoni, Venous and arterial thrombosis: two aspects of the same disease? Eur J Intern Med 20 (2009) 660–661.
[22] E. Previtali, P. Bucciarelli, S.M. Passamonti, I. Martinelli, Risk factors for venous and arterial thrombosis, Blood Transfus 9 (2011) 120–138.
[23] Berlin G, Hammar M, Tapper L & Tynngard N. Effects of age, gender and menstrual cycle on platelet function assessed by impedance aggregometry. Platelets DOI https://doi.org/10.1080/09537104.2018.1466387.
[24] R.F. Walker, E.E. Codd, Neuroimmunomodulatory interactions of norepinephrine and serotonin, J. Neuroimmunol 10 (1985) 41–58.
[25] N.T. Thompson, M.C. Scrutton, R.B. Wallis, Synergistic responses in human platelets. Comparison between aggregation, secretion and cytosolic Ca2+ concentration, Eur J. Biochem 161 (1986) 399–408.
[26] Brenner B, Harney JT, Ahmed BA, Jeffus BC, Unal R, Mehta JL, Kilic F. Plasma serotonin levels and the platelet serotonin transporter. J Neurochem 2007; 102(1): 206–15. Epub May 15.
[27] D. Shiffman, C.M. Rowland, J.Z. Louie, M.M. Luke, L.A. Bare, J.I. Bolonick, B.A. Young, J.J. Catanese, C.F. Stiggins, C.R. Pullinger, E.J. Topol, M.J. Malloy, J.P. Kane, S.G. Ellis, J.J. Devlin, Gene variants of VAMP8 and HNRPULI are associated with early-onset myocardial infarction, Arterioscler Thromb Vasc Biol 26 (2006) 1613–1618.
[28] A.D. Ma, C.S. Abrams, Pleckstrin induces cytoskeletal reorganization via Rac-dependent pathway, J Biol Chem 274 (1999) 28730–28735.

ANNEX 1 CAPÍTOL 4.2

La figura mostra els nivells de VAMP8, SERT i Serotonina en dones en edat fèrtil (<50 anys) i en menopàusiques i post menopàusiques (>50anys):



Trobem nivells més alts de VAMP8, SERT i serotonina en dones en edat fèrtil que en menopàusiques i postmenopàusiques.

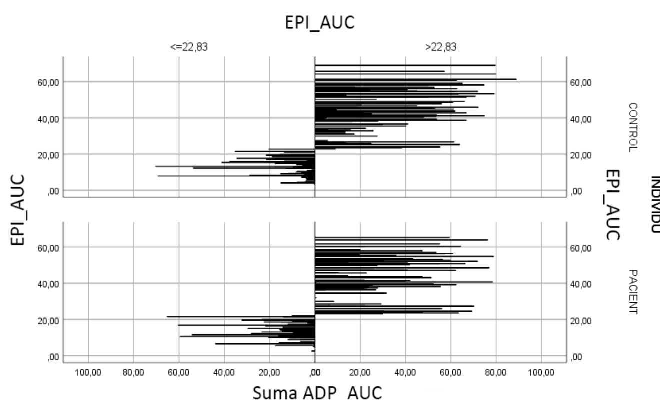
Per altra banda, no vam trobar correlació estadísticament significativa entre els nivells d'aquestes biomolècules i els nivells de PFA-100:

	PFA_ EPI	**PFA_ ADP**
VAMP8	-0,065	0,022
SERT	-0,103	- 0,113
SEROTONINA	0,063	0,038

ANNEX 2 CAPÍTOL 4.2

Vam classificar segons la reactivitat plaquetària els individus d'aquesta població (314 individus) a partir de la classificació obtinguda en la població RETROVE (capítol 4.1). Vam voler determinar si els nivells de VAMP8, SERT i serotonina s'associaven a trombosi en els individus amb hiperagregabilitat plaquetària (INDIVIDUS HIPER).

Vam definir els "individus Hiper" com els individus que eren hiper-ADP i/o hiper-EPI:



Vam trobar un 43% d'individus hipo (n=134) versus un 57 % d'individus hiper (n=180). En la població hipo el 60% eren controls (35 dones i 45 homes) i 40% eren pacients (27 dones i 27 homes). En la població hiper el 53% eren controls (63 dones i 32 homes) i 47 % eren pacients (49 dones i 36 homes).

Vam analitzar les biomolècules amb els mateixos criteris que a l'article d'aquest capítol 4.2 (percentil 75 de VAMP8 i SERT i el percentil 25 de serotonina). Vam fer una regressió logística per a calcular la OR de risc trombòtic crua (Model 1) i ajustada: primer es va ajustar per edat (Model 2) i després per edat, IMC, Factor V Leiden, FVIII i FvW (Model 3). Els valors p <0,05 es van considerar estadísticament significatius.

Vam obtenir els següents resultats:

		INDIVIDUS HIPO				INDIVIDUS HIPER			
VAMP8		Control		Pacient		Control		Pacient	
	<1930 (pg/10^9 PLT)	59 (74%)		34 (61%)		75 (78%)		57 (67%)	
	≥1930 (pg/10^9 PLT)	22 (26%)		21 (39%)		21 (22%)		28 (33%)	
	OR CRUA	1,65 (0,79- 3,44) ns				1,75 (0,90- 3,40) ns			
		DONES		HOMES		DONES		HOMES	
		Control	Pacient	Control	Pacient	Control	Pacient	Control	Pacient
	<1930 (pg/10^9 PLT)	29 (83%)	19 (70%)	30 (67%)	15 (52%)	55 (87%)	29 (59%)	20 (59%)	28 (78%)
	≥1930 (pg/10^9 PLT)	7 (17%)	8 (30%)	15 (33%)	13 (48%)	8 (13%)*	20 (41%)*	13 (41%)	8 (22%)
	OR CRUA	1,74 (0,54- 5,61) ns		1,73 (0,65- 4,56) ns		4,74(1,86- 12,08) (p=0,001)		0,43 (0,15- 1,25) ns	
	OR EDAT					3,80 (1,46-10,92) (p=0,007)			
	OR TOT					5,23 (1,53-17,89) (p=0,008)			
SERT		INDIVIDUS HIPO				INDIVIDUS HIPER			
		Control		Pacient		Control		Pacient	
	<784 (pg/10^9 PLT)	63 (77%)		39 (70%)		70 (74%)		48 (56%)	
	≥784 (pg/10^9 PLT)	18 (23%)		16 (30%)		26 (26%)*		37 (44%)*	
	OR CRUA	1,43 (0,65- 3,14) ns				2,07 (1,11- 3,86) (p=0,020)			
		DONES		HOMES		DONES		HOMES	
		Control	Pacient	Control	Pacient	Control	Pacient	Control	Pacient
	<784 (pg/10^9 PLT)	33(94%)	20(74%)	30(64%)	19(67%)	51 (81%)	30 (61%)	19 (60%)	18 (50%)
	≥784 (pg/10^9 PLT)	3(6%)	7(26 %)	15(36%)	9(33%)	12 (19%)*	19(39%)*	14 (40%)	18 (50%)
	OR CRUA	3,85 (0,89- 6,61) (ns)		0,95 (0,34- 2,59) ns		2,69 (1,15- 6,31) (p=0,021)		1,35 (0,52- 3,51) ns	
	OR EDAT					2,79 (1,07-7,25) (p=0,035)			
	OR Edat BMI					2,53 (0,96-6,66) (p=0,059)			
	OR TOT					2,15 (0,70-6,61) ns			

		INDIVIDUS HIPO				INDIVIDUS HIPER			
		Control		Pacient		Control		Pacient	
SEROTONINA	>115 (ng/10^9 PLT)	60 (75%)		34 (67%)		70 (76%)		45 (55%)	
	≤115 (ng/10^9 PLT)	19 (25%)		18 (33%)		22 (24%)*		37 (45%)*	
	OR CRUA	1,28 (0,64- 2,53) ns				2,17 (1,22- 3,08) (p=0,006)			
		DONES		HOMES		DONES		HOMES	
		Control	Pacient	Control	Pacient	Control	Pacient	Control	Pacient
	>115 (ng/10^9 PLT)	24 (66%)	17 (65%)	36 (82%)	17 (68%)	44(75%)	19(40%)	26 (0%)	26 (0%)
	≤115 (ng/10^9 PLT)	11 (34%)	9 (35%)	8 (18%)	9 (32%)	15 (25%)*	29 (60%)*	7(100%)	8(100%)
	OR CRUA	1,07 (0,41- 2,81) ns		1,49 (0,56- 3,94) ns		3,97 (1,86- 8,47) (p=0,0001)		0,8 (0,29- 2,26) ns	
	OR EDAT					3,08 (1,27-7,45) (p=0,013)			
	OR TOT					4,10 (1,36-12,37) (p=0,012)			

Es mostra els càlculs per al percentil 75 de VAMP8 i SERT i el percentil 25 de SEROTONINA en homes i dones.
*resultats significativament diferents.
Model 1: model no -ajustat.
Model 2: ajustat per edat.
Model 3 (TOT): ajustat per edat, IMC, Factor V Leiden, FVIII i FvW.

Vam trobar diferències estadísticament significatives entre els controls i els pacients amb els nivells de VAMP8, SERT i serotonina en les dones amb hiperagregabilitat plaquetària, no així en els individus (homes i dones) amb hipoagregabilitat plaquetària.

CAPÍTOL 4.3

LOW ADAMTS13 LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN WOMEN.

CAPÍTOL 4.3

LOW ADAMTS13 LEVELS ARE ASSOCIATED WITH VENOUS THROMBOSIS RISK IN WOMEN.

RESUM:

El nostre objectiu va ser determinar si nivells baixos d’ ADAMTS13 incrementaven el risc de trombosi venosa i si l’ADAMTS13 era depenen del grup sanguini en població espanyola. Vam estudiar 250 pacients i 250 controls en un estudi de casos –controls. No vam trobar diferències en les mitjanes dels nivells d’ADAMTS13 entre pacients i controls. Un percentatge més alt de pacients tenien nivells per sota del percentil 5 i 10. Quan els individus amb el percentil 5 es van estudiar per gènere, es va obtenir una diferència estadísticament significativa només en les dones. El risc trombòtic va ser de 4,34 (1,3-13,4 IC 95%). Aquest risc no va canviar quan es va incloure les covariables en l’anàlisi. També, vam trobar que els nivells de l’ADAMTS13 no mostraven diferències entre els grups sanguinis. Els nivells del factor von Willebrand en els grups sanguinis A, B i AB eren significativament diferents dels nivells en el grup O tant en pacients com en controls. Els nivells de factor von Willebrand en els individus que eren A_2O eren similars als del grup OO. No es va trobar interacció en els nivells de factor von Willebrand entre pacients i controls o entre sexes, però en canvi es va trobar una correlació entre el factor von Willebrand i l’edat. En conclusió, vam trobar que els nivells baixos de ADAMTS13 són un risc independent de trombosi venosa en dones espanyoles i els nivells d’ADAMTS13 són independents del grup sanguini. Els nivells de factor von Willebrand relacionats amb el grup sanguini ABO en la nostra població s’incrementen amb el següent ordre: $O=A_2O<A_1<B<AB$.

Thrombosis Research 157 (2017) 38–40

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Letter to the Editors-in-Chief

Low ADAMTS13 levels are associated with venous thrombosis risk in women

Keywords:
ADAMTS13
von Willebrand factor
Thrombosis
ABO blood groups

Reduced levels of plasma ADAMTS13 (a disintegrin and metalloprotase with thrombospondin type 1 motif) and an increase of plasma von Willebrand factor (vWf) concentration are risk factors for arterial thrombosis [1]. There is an association between high levels of vWf and venous thrombosis [2]. Also, high levels of ADAMTS13 activity are related to venous thrombosis [3]. Also, ADAMTS13 and vWf levels are associated with venous thromboembolism in patients with cancer [4]. In our study, we aimed to determine if low levels of ADAMTS13 increases the risk of venous thrombosis and also if ADAMTS13 distribution is dependant on the ABO blood groups in a Spanish population.

Patients and controls were recruited from November 1997 to April 2002 in our Thrombosis and Haemostasis Unit. Our case-control study has been described previously [5]. It included 250 patients and 250 controls. We obtained plasma from 214 patients (93 males, 121 females) and 224 controls (98 males, 126 females). The patients were included if they had suffered their first thrombotic event when under 70 years of age. Overall, 47% of patients had spontaneous thrombosis and 53% had secondary thrombosis (44% of fertile women were using oral contraceptives). Deep vein thrombosis was found in 59% and pulmonary embolism in 31%, while upper arm thrombosis in 8% and intracranial sinus thrombosis in 2%. Each patient recruited his/her own healthy control with the following criteria: similar age (±10 years), same sex, no genetic relationship to the patient and no personal or family history of venous thrombosis. All procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau in Barcelona. Written informed consent was obtained from all participants.

Blood was collected 6 months after the most recent thrombotic event. Antivitamin K administration was withdrawn. The samples were taken after a washout period of at least 20 days. The ADAMTS13 level was determined in 2010; samples were stored at −80 °C until tested. To diminish the possible instability of the samples, the ADAMTS13 antigen was determined by TECHNOZYM®ADAMTS13 ELISA (Technoclone GmbH, Vienna, Austria) according to the manufacturer's instructions. The vWf Antigen was determined by the commercial kit Von Willebrand Factor Antigen test REAADS (Broomfield, Colorado, USA). The ADAMTS13 antigen was recorded in μg/mL (variation coefficient: 5.4% for intra-assay and 6.8% for inter-assay). von Willebrand antigen levels were recorded as percentages of a Reference plasma (variation coefficient: 3.6% for intra-assay and 5.0% for inter-assay). The assays for Thrombotic factors have been described previously [5]. They included antithrombin, Protein C, total and free Protein S, FVIII clotting activity, activated Protein C resistance and Factor V Leiden (FVL). DNA was isolated from peripheral blood leukocytes by a standard protocol. Blood group genotyping was performed by the polymerase chain reaction (PCR) and subsequent digestion as previously described [6]. We were able to discriminate among A1, A2, O1, O2 and B alleles. The Student *t*-test was used to calculate the mean differences between groups (values are expressed as mean and 95% confidence intervals). We performed the χ^2 test for group comparison of frequencies (p values < 0.05 were considered statistically significant) A logistic regression method was used to estimate both the crude and adjusted odds ratio (OR) with 95% confidence intervals. Also, adjustments were made for sex, age, FVL, 20210A prothrombin mutation, 46C → T of the F12 gene variant, levels of Factor VIII > 90th percentile and ABO blood group genotype. We performed an analysis of variance to determine if there was interaction between gender and ADAMTS13 and the risk of thrombosis. The plasma levels of vWf and ADAMTS13 were compared among the different blood groups by ANOVA. An analysis of variance by two factors was done to determine the vWf distribution depending on the ABO genotype by individual group. Multiplex contrasts were applied (Bonferroni's post-hoc). Pearson's coefficient correlation was calculated between age and vWf levels. For vWf differences related to ABO type by age, an ANOVA by two factors was applied. For statistical analyses, the SPSS 21.0 software was used.

1. ADAMTS13 and venous thrombosis

The normal range of ADAMTS13 levels was 0.50 μg/mL to 1.82 μg/mL. No significant mean differences of ADAMTS13 levels were found between patients and controls (1.15 μg/mL vs 1.16 μg/mL; $p = 0.800$). ADAMTS13 levels were stratified into: high levels (above 95th percentile), intermediate levels (between 5th and 95th percentile) and low levels (below 5th percentile). Statistical differences were found only between patients and controls with low levels. A higher percentage of patients had levels under percentiles 5th (67.6% vs 32.4%; difference 35.2%, 95%CI: 26.4–44, $p = 0.017$) and 10th (62.3% vs 37.7%; difference of 24.6, 95%CI: 15.5–33.7, $p = 0.024$). When individuals in the 5th percentile were studied by gender, a significant statistical difference was obtained, but only in women: 78.9% vs 21.1%; difference of 57.8%; 95%CI: 47.6–68, $p = 0.007$. The associated thrombotic OR was 4.31 (1.3–13.4 95%CI). The OR did not change when covariates were included (OR = 4.2 (1.28–13.8 95%CI)) (Table 1). Importantly, males did not show significant differences between patients and controls as is shown in Table 1. The variables sex, ADAMTS13 and thrombosis risk did not show any interaction.

ADAMTS13 levels were not different between users of contraceptives and non-users.

http://dx.doi.org/10.1016/j.thromres.2017.07.001
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Table 1
5th percentile and 10th percentile of ADAMTS13 in women and men.

		5th percentile ADAMTS13				10th percentile ADAMTS13				Total
		>0.69 (μg/mL)	≤0.69 (μg/mL)	OR (95%CI)	OR adjusted (95%CI)	>0.78 (μg/mL)	≤0.78 (μg/mL)	OR (95%CI)	OR adjusted (95%CI)	
Women	Control	122 (54%)	4 (21%)*	4.31 (1.3–13.4)	4.2 (1.3–13.8)	115 (54%)	11 (32%)*	2.54 (1.1–2.0)	2.69 (1.1–6.4)	126
	Patient	106 (46%)	15 (79%)*			98 (46%)	23 (68%)*			121
Men	Control	90 (52%)	8 (44%)			86 (52%)	12 (44%)			98
	Patient	83 (48%)	10 (56%)			78 (48%)	15 (56%)			93

* Significant different results. Normal range: (0.50–1.82) μg/mL.

2. ADAMTS13, vWf and ABO blood group genotype

We found that ADAMTS13 levels were not significantly different among blood groups either in patients or in controls as is shown in Fig. 1a.

In contrast the vWf levels in blood groups A, B and AB were significantly higher than blood group O except for A_2O in both patients and controls (Fig. 1b). The mean differences of total individuals among group O and group A_1 were: 46.07 (62.52 to 29.63) $p = 0.0005$, between group O and group B were: 56.68 (81.00 to 32.35) $p = 0.0005$ and

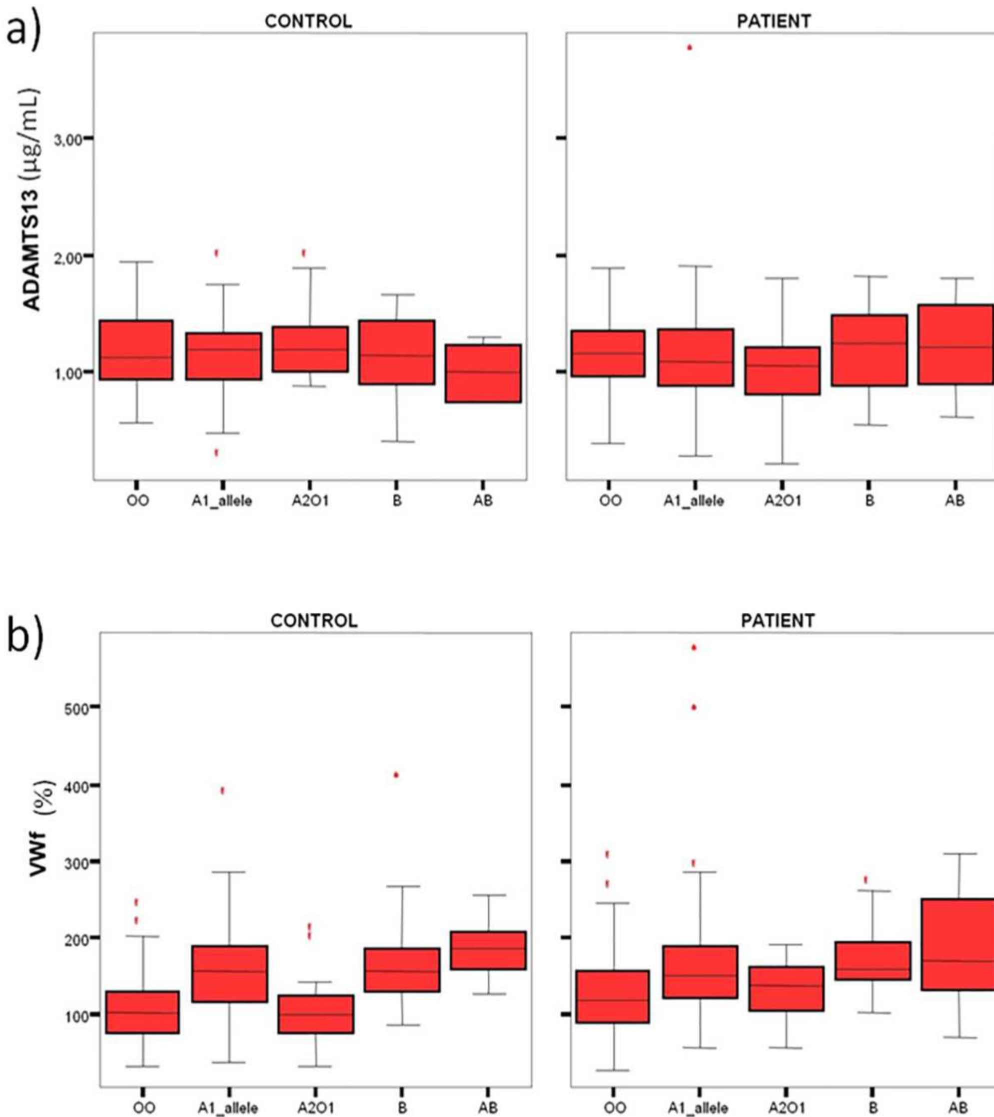

Fig. 1. ADAMTS13 (a) and vWf (b) levels depending on ABO blood group in patients and controls.

between group O and group AB were: 73.27 (112.77 to 33.76) $p = 0.0005$. Finally, among group O and A_2O group no significant difference in means was observed: 1.60 (34.14 to30.94) $p = 1.000$. No interaction was found for vWf levels between patients and control or between the two sexes, but a correlation between vWf and age was found ($R = 0.331$). We found that 11% of vWf levels (R^2) depended on age. Individuals older than 50 years had higher levels of vWf than individuals younger than 50. Mean difference 35%. Both groups followed the same blood group profile: $O = A_2O < A_1 < B < AB$.

An important result of our study is that in a Spanish population, low levels of ADAMTS 13 are associated with high venous thrombotic risk, but only in women. In contrast, Mazzetto [4] observed increased levels of vWf associated with high levels of ADAMTS13 in patients with venous thromboembolism. Unfortunately, in this study the levels of ADAMTS13 were not evaluated by gender. Pépin [4] found that vWf levels were significantly higher in more advanced cancer and were positively associated with Vienna CATS score, whereas ADAMTS13 activity levels were inversely correlated. When ADAMTS13 activity and F1 + 2 levels were included, a considerable increase in the predictive value of these scoring systems for venous thrombosis was noted. Our study was completed before Pépin's study was published. Also, we determinate the ADAMTS13 antigen not its activity. However antigen and activity levels of ADAMTS13 are highly correlated ($R = 0.75$).

No other studies, as far as we know, have found an association between low levels of ADAMTS13 and high venous thrombosis risk in women. Little is known about ADAMTS13 levels and arterial thrombosis: In the SMILE [7] study, only with men, a protective effect of ADAMTS13 on myocardial infarction was found. Maino [8] found in an individual patient data meta-analysis on plasma ADAMTS13 levels that the risk of myocardial infarction was different between women and men. A similar risk difference between sexes with low versus high ADAMTS13 levels resulted in a higher relative risk in women than in men.

The RATIO [9] study reported that individuals with low ADAMTS13 levels who use contraceptives were approximately 5 times more likely to develop ischemic stroke than non-users. However, the risk of myocardial infarction in patients with low ADAMTS13 levels was not increased with contraceptives use. In our study, we did not find any association between contraceptive use and the risk of venous thrombosis among individuals with 5th or 10th percentile of ADAMTS13. Whether or not estrogen plays a role in this phenomenon is unknown.

We found that vWf levels were blood group dependent while ADAMTS13 levels were not. It has been hypothesized [10] that the lower levels of vWf in group O individuals could be a reflection of the high levels of ADAMTS13, but we agree with Al-Awadhi [10], who did not find these results. Also, ADAMTS13 levels did not differ among the different blood groups. These findings suggest that whatever is causing lower levels of vWf is not related to quantitative changes in ADAMTS13, but may be related to the structural difference in the vWf protein in individuals of different blood groups.

Our study has some limitations. Primarily because we determined the ADAMTS13 antigen and not its activity, it is possible that ADAMTS13 activity would be more informative. Second, could be the mean freezing time but it is estimated that ADAMTS13 antigen loses about 0.06% activity per year (WHO/BS/3014.2246). Third, since we are a reference center in our region, it is possible that the patients we studied represented a sample with higher thrombotic risk than a random group of patients. Finally the number of patients and controls may not have provided enough statistical power. Further studies with a larger population may support our results.

In conclusion, low levels of ADAMTS13 are an independent risk factor for venous thrombosis in Spanish women. Also, vWf levels are blood group dependent while ADAMTS13 levels are not. We observed that A_2O genotype gave similar results as the O genotype with vWf. The order of vWf levels that depend on the ABO genotype was $O = A_2O < A_1 < B < AB$. Finally, the vWf distribution depending on ABO group is independent of sex and individual group (case-control).

Sources of funding

Spanish grants: RIC, RD12/0042/0032.

Conflict of interest

None.

Acknowledgements

We would like to thank Professor William Stone and Dr. Montserrat Borrell for reviewing the manuscript.

References

[1] Michelle A.H. Sonneveld, Moniek P.M. de Maat, Frank W.G. Leebeek, Von Willebrand factor and ADAMTS13 in arterial thrombosis: a systematic review and meta-analysis, Blood Rev. 28 (2014) 167–178.

[2] J.C. Souto, L. Almasy, M. Borrell, F. Blanco-Vaca, J. Mateo, J.M. Soria, I. Coll, R. Felices, W. Stone, J. Fontcuberta, J. Blangero, Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study, Am. J. Hum. Genet. 67 (2000) 1452–1459.

[3] B.M. Mazetto, F.L. Orsi, A. Barnabé, E.V. De Paula, M.C. Flores-Nascimento, J.M. Annichino-Bizzacchi, Increased ADAMTS13 activity in patients with venous thromboembolism, Thromb. Res. 130 (2012) 889–893.

[4] M. Pépin, A. Kleinjan, D. Hajage, H.R. Büller, M. Di Nisio, P.W. Kamphuisen, L. Salomon, A. Veyradier, A. Stepanian, I. Mahé, ADAMTS13 and von Willebrand factor predict venous thromboembolism in patients with cancer, J. Thromb. Haemost. 14 (2016) 306–315.

[5] I. Tirado, J.M. Soria, J. Mateo, A. Oliver, J.C. Souto, A. Santamaria, et al., Association after linkage analysis indicates that homozygosity for the 46C>T polymorphism in the F 12 gene is a genetic risk factor for venous thrombosis, Thromb. Haemost. 91 (2004) 899–904.

[6] J.C. Souto, L. Almasy, E. Muniz-Díaz, et al., Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2024–2028.

[7] C.K. Chion, C.J. Doogen, J.T. Crawley, D.A. Lane, F.R. Roseendal, ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men, Blood 109 (2007) 1998–2000.

[8] A. Maino, B. Siegerink, L.A. Lotta, J.T.B. Crawley, S. Le Cessie, F.W.G. Leebeek, D.A. Lane, G.D.O. Lowe, F. Peyvandi, F.R. Rosendaal, Plasma ADAMTS-13 levels and the risk of myocardial infarction: an individual data meta-analysis, J. Thromb. Haemost. 13 (2015) 1396–1404.

[9] H.M. Andersson, B. Siegerink, B.M. Luken, J.T. Crawley, A. Algra, D.A. Lane, F.R. Rosendaal, High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women, Blood 119 (2012) 1555–1560.

[10] Anwar M. Al-Awadhi, Seham K. Al-Sharrah, Mehrez M. Jadaon, Faisal Al-Sayegh, Investigating the influence of age, gender and ABO blood group on ADAMTS-13 antigen and activity levels in healthy Arabs, Blood Transfus. 12 (2014) 138–140.

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22 March 2017

ANNEX 1 CAPÍTOL 4.3

Original complert de l'article enviat (va ser acceptat com la carta a l'Editor que s'ha mostrat en el capítol 4.3):

TITLE: **LOW ADAMTS13 LEVELS INCREASE VENOUS THROMBOSIS RISK IN WOMEN.**

THROMBOSIS RESEARCH

Title page (short title): Low ADAMTS13 levels increase venous thrombosis risk in women

LOW ADAMTS13 LEVELS INCREASE VENOUS THROMBOSIS RISK IN WOMEN.

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LOW ADAMTS13 LEVELS INCREASE VENOUS THROMBOSIS RISK IN WOMEN.

ABSTRACT:

Our objective was to determine if decreased levels of ADAMTS13 increased the risk of venous thrombosis and if ADAMTS13 was blood group dependent in the Spanish population. We studied 250 patients and 250 controls in a case-control study. No significant mean differences of ADAMTS13 levels were found between patients and controls. A higher percentage of patients had levels under percentiles 5th and 10th. When individuals in the 5th percentile were studied by gender, a significant statistical difference was obtained only in women. The thrombotic OR was 4.31 (1.3-13.4 95%IC). The risk did not change when covariates were included in the analysis. Also, we found that ADAMTS 13 levels did not show a difference among the blood groups. The von Willebrand factor levels in blood groups A, B and AB were significantly different from the levels in blood group O in both patients and controls. Von Willebrand factor levels in individuals who were A_2O were similar to those in the OO group. No interaction was found in von Willebrand factor levels between patients and control or between the two sexes, but a correlation between von Willebrand factor and age was found. In conclusion, we found that a low level of ADAMTS13 is an independent risk factor for venous thromboembolism in Spanish women and ADAMTS13 levels were independent of the blood group. The von Willebrand factor levels related to the ABO blood groups in our population were in the following increasing order: $O=A_2O<A_1<B<AB$.

Keywords: ADAMTS 13, von Willebrand factor, thrombosis, ABO blood groups.

Highlights:

1. ADAMTS13 could be an independent risk factor for venous thromboembolism in the Spanish female population

2. Von Willebrand factor is blood group dependent while ADAMTS13 is not.

3. Von Willebrand factor level is related to the ABO blood groups in the Spanish population in the following increasing order: $O=A_2O<A_1<B<AB$

INTRODUCTION:

The Von Willebrand factor (vWf), as a marker of endothelial dysfunction and platelet activation, plays a crucial role in platelet adhesion and thrombus formation[1]. It is stored as a multimer in platelets and endothelium from where it is released during injury or inflammation. Decreased plasma concentrations of vWf are associated with the bleeding disorder called "von Willebrand disease". High plasma concentrations of vWf are related to an increase risk of thrombosis. Several studies[1-4] have demonstrated that this association occurs primarily with the risk of arterial thrombosis, such as coronary heart disease and ischemic stroke.

Approximately 30% of the genetic variation that influences the levels of vWf in plasma is influenced by the ABO blood groups. Many reports[5-7] have described the influence of the ABO blood groups on plasma vWf and FVIII levels. We found also that the ABO blood groups and factor VIII levels were independent risk factors for venous thromboembolism[8]. Individuals of blood group O have approximately 25% lower levels of vWf than individuals of non-O[7,9] blood group. Genotypic analysis confirmed these previous findings, and identified the highest vWf levels among A_1A_1, *BB* and A_1B individuals[10-13]. Several hypotheses have been suggested to explain why group O individuals have low levels of vWf. For example, the ABO blood group could affect the rate of synthesis/secretion of vWf. Also blood group O individuals might affect the survival of the protein and its clearance from the plasma.

The antigens of the ABO blood groups (A, B and H determinants) consist of complex carbohydrate molecules[14]. The *A* and *B* alleles encode A and B glycosyltransferase activities which convert the precursor H antigen into either A or B determinants. The *A* allele adds N-acetylgalactosamine and the *B* allele adds D-galactosamine to the H antigen. Blood group O individuals lack such transferase enzymes and, consequently continue to express the H molecule constituting a solitary terminal fucose moiety. A single base deletion in the *A* gene is expressed as the A_2 allele. Also, as a result of a frameshift mutation the glycosyltransferase coded by A_2 allele has an additional carboxyterminal domain which seems responsible for the drastic reduction in enzyme activity[15].

ADAMTS13 (a disintegrin and metalloprotase with thrombospondin type 1 motif) regulates the size of the vWf multimer. It degrades the multimer into smaller and less active forms cleaving vWf at Tyr1605-Met1606 peptide bond. ADAMTS13 reduces platelet adhesion and aggregation. Recent studies[16] demonstrated its anti-inflammatory effects as well. Low levels of ADAMTS13 activity (by deficiency or by inhibitor) causes the circulation of ultra-large vWf multimers that promote platelet aggregation and micro-vascular thrombi leading to thrombotic thrombocytopenic purpura (TTP).[17-18]

It has been demonstrated that reduced plasma ADAMTS13 activity and increased plasma vWf concentration are risk factors for myocardial infarction[19-23], ischemic stroke[24-26], pre-eclampsia[27-28], and malignant (or cerebral) malaria[29-33]. The association between vWf and ADAMTS13 with arterial thrombosis is summarized in the review by Sonneveld[34]. There is an association between high levels of vWf and venous thrombosis[35-38]. Also high levels of ADAMTS13 activity and venous thrombosis[39] have been reported. Recently, Maino[40] did a meta-analysis with more than 1500 individual patients from the following studies: SMILE, Milan, RATIO, GLAMIS and ATTAC). The studies were a case-control design. The results were contradictory regarding the association of low ADAMTS13 levels with myocardial infarction. In fact a protective effect (SMILE study)[41], no effect (Milan study)[42], and a risk-increasing effect (RATIO, GLAMIS, ATTAC studies)[19-23].

An important role has been described for vWf and the ABO carbohydrate structures as their ability to regulate vWf levels and the susceptibility to proteolysis by ADAMTS13. The vWf susceptibility to ADAMTS13 appears to decrease in the order O>B>A≥AB[43]. This effect on the rate of vWf cleavage by ADAMTS13 has been demonstrated also[44-46], where the alteration of vWf glycosylation significantly affected ADAMTS13–dependent vWf proteolysis.

In our study, we aimed to determine if low levels of ADAMTS13 increases the risk of venous thrombosis and also if ADAMTS13 distribution is dependant on the ABO blood groups in the Spanish population.

MATERIAL AND METHODS:

Study design

Patients and controls were recruited in our Thrombosis and Haemostasis Unit. Our unit is a reference center for several hospitals in our region. Patients were included when they were referred to our hospital from November 1997 to April 2002. Our case-control study has been previously described[8,47]. From the initial case-control study that included 250 patients and 250 controls, we analyzed 214 patients (93 males, 121 females) and 224 controls (98 males, 126 females). The patients were included in our study if they had suffered their first thrombotic event when under 70 years of age. Their clinical characteristics are shown in Table 1. Each patient was asked to find his/her own healthy control according to the following criteria: similar age (±10 years), same sex, no genetic relationship to the patients and no personal or family history of venous thrombosis. Some partners of patients joined the study as control subjects. All procedures were approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau in Barcelona. Written informed consent was obtained from all participants.

	n	%
Family history of thrombosis	83	38.8
Age at first thrombosis (mean±SD)	42.8±14	
Multiple thrombosis	53	24.8
Spontaneous	101	47.2
Secondary*	113	52.8
general surgery	7	3.3
orthopedic surgery	15	7.0
abdominal surgery	10	5.0
gynecological surgeryǂ	5	4.1
immobilization	42	19.6
pregnancyǂ	17	23.9
oral contraceptivesŁ	31	44.0
hormonal replacement therapyǂ	5	3.6
indwelling catheters	3	1.4
autoimmune disease	2	0.9

varicose veins	2	0.8
paralyzed legs	1	0.4
Others	10	4
Site of thrombosis		
deep vein thrombosis	126	58.9
pulmonary embolism§	66	30.8
upper arm thrombosis	17	7.9
intracranial sinus thrombosis	5	2.3

*Some patients had more than one risk factor (% of all cases); ɨ only women were considered; Ⱡ only fertile women were considered (15-45 years); §deep venous thrombosis was diagnosed in 38 patients with pulmonary embolism.

Table 1. Patients' clinical characteristics.

Blood collection

Blood was collected within 6 months after the most recent thrombotic event. Antivitamin K administrations were withdrawn. The samples were taken after a washout period of at least 20 days. Blood samples were collected from the antecubital vein and immediately anticoagulated with 1/10 volume of 0.129M sodium citrate. Platelet-poor plasma was obtained by centrifugation at 2000 g for 20 min and frozen and stored at – 40ºC until analyzed.

Laboratory determinations

The ADAMTS13 antigen was determined by TECHNOZYM®ADAMTS13 ELISA (Technoclone GmbH, Vienna, Austria) according to the manufacturer's instructions. The vWf Antigen was determined by the commercial kit Von Willebrand Factor Antigen test REAADS (Broomfield, Colorado, USA).

The ADAMTS13 antigen was recorded in µg/mL and as percentages of a Reference plasma. The intra-assay coefficient of variation was estimated to be 5,4% and the inter-assay coefficient of variation was estimated to be 6,8%. Von Willebrand antigen levels were recorded also as percentages of a Reference plasma. The intra-assay variation was 3,6 % and inter-assay coefficients was 5,0% .

The assays for Thrombotic factors have been described previously in detail[47]. They included antithrombin, Protein C, total and free Protein S, FVIII clotting activity, activated Protein C resistance (APCR) and Factor V Leiden.

Genetic analysis

DNA was isolated from peripheral blood leukocytes by a standard protocol[48]. Blood group genotyping was performed by the polymerase chain reaction (PCR) and subsequent digestion as previously described[12]. This allowed us to discriminate among A_1, A_2, O_1, O_2 and *B* alleles.

Statistical analysis

The Student t test was used to calculate the mean differences between groups (values are expressed as mean and 95 % confidence intervals). We performed the Chi-square test (χ^2) for group comparison of frequencies (*P* values less than 0.05 were considered statistically significant) A logistic regression method was used to estimate both the crude and adjusted odds ratio (OR) with 95% confidence intervals. Adjustments were made for sex, age and those factors previously associated with venous thrombosis in our population, including: FVL and levels of FVIII>90th percentile[8,47]. The plasma levels of vWf and ADAMTS13 were compared among the different blood groups by ANOVA. For the vWf distribution depending on *ABO* genotype by individual group, an analysis of variance by two factors was done. Multiplex contrasts were applied (Bonferroni's post-hoc). Pearson's coefficient correlation was calculated between age and vWf levels. For vWf differences related to ABO type by age, an ANOVA by two factors was applied. vWf/ADAMTS13-ratio was calculated as follows: %vWf/100x(ADAMTS13 concentration in µg/ml). For statistical analyses, the SPSS 21.0 software was used.

RESULTS:

ADAMTS13 and venous thrombosis:

No significant mean differences of ADAMTS13 levels were found between patients and controls (1.1510 vs 1.1599; p=0.800).

Individuals with levels of ADAMTS13 below the 5th percentile (≤0.69 μg/mL) and 10th percentile (≤0.78 μg/mL) were selected. A percentage of patients higher than controls were observed below the 5th percentile (67.6% vs 32.4%; difference 35.2%, 95%CI: 26.4-44, p=0.017) as well as below 10th percentile (62.3% vs 37.7%; difference of 24.6, 95%CI: 15.5-33.7, p= 0.024).
When the distribution below the 5th percentile was studied by gender, a significant statistic result was obtained in women: 78.9% vs 21.1%; difference of 57.8%; 95%CI: 47.6-68, p=0.007 (Table2). The associated thrombotic OR was 4.31 (1.3-13.4 95%CI). The OR did not change when covariates were included, the OR obtained was 4.2 (1.28-13.8 95%CI). Importantly, males did not show significant differences among patients and controls as is shown in Table 3.

WOMEN	**5th Percentile ADAMTS13**		**10th Percentile ADAMTS13**		
	>0.69	≤0.69	>0.78	≤0.78	TOTAL
CONTROL	122(54%)	4(21%)*	115(54%)	11(32%)*	126
PATIENT	106(46%)	15(79%)*	98(46%)	23(68%)*	121

Table 2. 5th percentile and 10th percentile of ADAMTS13 in women. *Significant different results.

MEN	**5th Percentile ADAMTS13**		**10th Percentile ADAMTS13**		
	>0.69	≤0.69	>0.78	≤0.78	TOTAL
CONTROL	90(52%)	8(44%)	86(52%)	12(44%)	98
PATIENT	83(48%)	10(56%)	78(48%)	15(56%)	93

Table 3. 5th percentile and 10th percentile of ADAMTS13 in men.

When men were added to the logistic regression model, significant differences of ADAMTS13 were not found.

We calculated the 5th and 10th percentile of women and men separately. We obtained the same results: significant results in women versus non significant results in men (results not shown).

We did not find significant differences between patients and controls in women who used contraceptive and non-users in 5th percentile or 10th percentile of ADAMTS13. When contraceptives were added as a covariate to the logistic regression model, it was not a confusing variable.

ADAMTS13, vWf and ABO blood group genotype:

We find that ADAMTS13 levels did not exhibit significant differences among blood groups either in patients or in controls as is shown in Figure 1

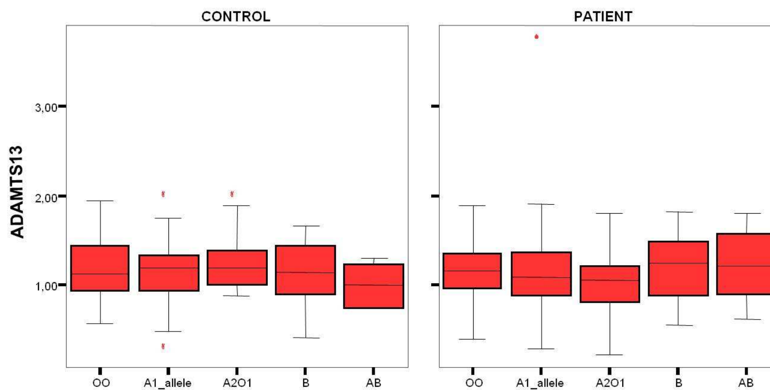


Figure 1. ADAMTS13 levels depending on ABO blood group in patients and controls

The Wf levels in blood groups A, B and AB were significantly higher than blood group O except for A_2O in both patients and controls (Figure 2) as it shown with the mean differences of total individuals among group O and group A_1: 46.07 (62.52 to 29.63) p=0.0005, group B: 56.68 (81.00 to 32.35) p=0.0005 and group AB: 73.27 (112.77 to 33.76) p= 0.0005. Finally, among group O and A_2O group no significantly difference in means was observed: 1.60 (34.14 to30.94) p=1.000.

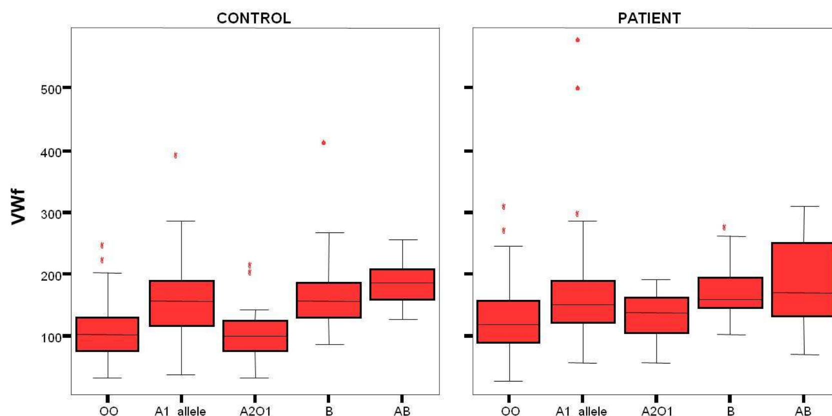


Figure 2. vWf levels depending on ABO blood group in patients and controls

We observed also that the vWf/ADAMTS13 ratio was the same profile as vWf levels in both patients and controls (results not shown).
vWf distribution related to *ABO* genotype was independent of the individual group (case-control) as is shown in Figure 3.

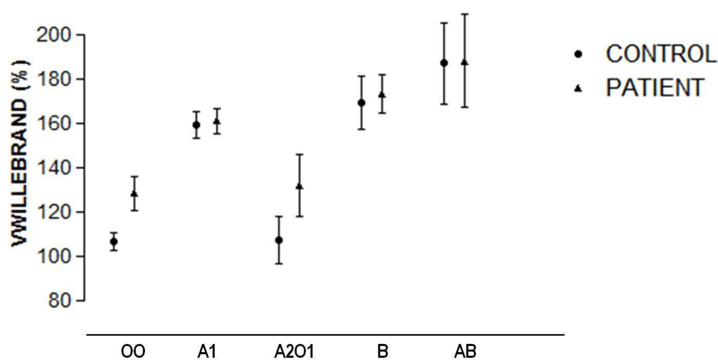


Figure 3. vWf distribution depending on *ABO* genotype by individual group (case-control).

As no relation was found in vWf levels within the case-controls we showed mean values for vWf of total individuals (patients and controls): The mean values for vWf were higher in A_1 blood group (160% (CI 95% 152-169), B group (171%(CI 95% 156-185)) and also AB group (187% (CI95% 156-219)) than in O group (114% (CI95% 107-122)). The vWf levels in A_2O genotype were similar to the *OO* genotype (116% (CI95% 88-134)).

We found a significant correlation between vWf levels and age (R=0.331). 11% of vWf levels (R^2) depended on age. Individuals older than 50 years were considered old in contrast to individuals less than 50 as young individuals. We found that the young had lower levels of vWf than the old individuals, but both followed the same profile: $O=A_2O<A_1<B<AB$. A 35 % mean difference was observed between people older and younger than 50 years as is shown in Figure 4.

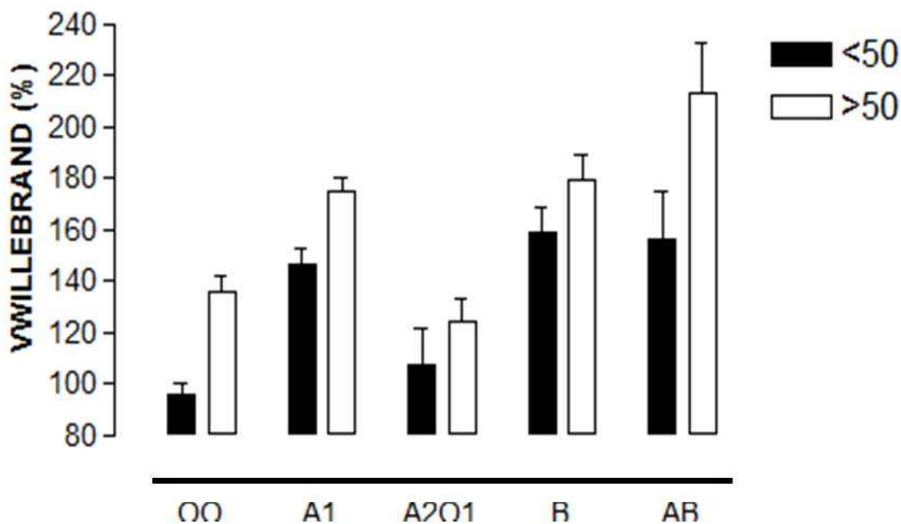


Figure 4. vWf distribution related with *ABO* genotype by age.

No differences were found between vWf and sex.

DISCUSSION:

The most important result of our study is that in the Spanish population, low levels of ADAMTS 13 are associated with high venous thrombotic risk, but only in women. In contrast, Mazzetto[39] observed increased levels of vWf associated with high levels of ADAMTS13 in patients with venous thromboembolism. This unexpected result could be due to increased levels of ADAMTS13 which could compensate for the high levels of vWf. This study analyzed the relationship between inflammation and venous thrombosis and found that Tumor Necrosis factor-α and Interleukin-6 are increased in patients. This suggests that such

patients may present increased levels of inflammatory markers even a long time after the acute venous thrombosis episode. Unfortunately, the levels of ADAMTS13 were not evaluated by gender. No other studies, as far as we know, have found the association between low levels of ADAMTS13 and high venous thrombosis risk in women. There is a little known about ADAMTS13 levels and arterial thrombosis:

In the SMILE study[41], which was made only with men, a protective effect of ADAMTS13 on myocardial infarction was found.

Maino[40] found in his individual patient data meta-analysis on plasma ADAMTS13 levels that the risk of myocardial infarction was different between women and men. Subgroup analysis showed that the association between ADAMTS13 levels and the risk of myocardial infarction was similar for men and women at all ages. However, the affect seemed to be more pronounced in women. This difference between women and men in relative risk could be from the division of the studies into two subgroups (i.e. SMILE, GLAMIS and ATTAC for men, and GLAMIS, RATIO, Milan and ATTAC for women). Also it could have resulted from the fact that myocardial infarction is higher in men than in women[49]. A similar absolute risk difference between sexes for low versus high ADAMTS13 levels will lead to a higher relative risk in women than in men.

We analyzed contraceptive influence on women. We did not find any association between contraceptive use and the risk of venous thrombosis among individuals with 5th or 10th percentile of ADAMTS13. In the RATIO study[19], it was found that individuals with low ADAMTS13 levels who use contraceptives were approximately 5 times more likely to develop ischemic stroke than non-users. However, the risk of myocardial infarction (and not ischemic stroke) in patients with low ADAMTS13 levels was not increased by the use of contraceptives.

Our study showed that vWf levels were blood group dependent while ADAMTS13 levels were not. Many reports[50] have confirmed the relationship between vWf and blood groups but few studies have demonstrated the relationship between ADAMTS13 and blood groups[51]. It has been hypothesized that the lower levels of vWf in group O individuals could be a reflection of the high levels of ADAMTS13, but we agree with *Al-Awadhi*[51], who did not find these results. Also, ADAMTS13 levels did not differ among the blood groups.

These findings suggest that whatever is causing lower levels of vWf is not related to quantitative changes in ADAMTS13, but may be related to the structural difference in the vWf protein in individuals of different blood groups.

We found that individuals with the *A_2O* genotype had the similar vWf levels as those who were Group O. This agrees with our previous findings[8] between *ABO* genotype and FVIII levels as well as with other authors[10]. *O'Donell et al.* reported that the level of the A-transferase activity detected in A_2O persons was low. The *A_2* allele differs from the *A_1* allele by a deletion of the normal stop codon. This A-transferase is a larger molecule (because it has an additional carboxyterminal domain) and is less efficient in converting the basic structures into A blood group. They observed that the amount of an antigen expressed per unit of vWf was significantly higher in *A_1A_1* and *A_2O* than in *OO* individuals. We found that the different vWf levels in our population as in the follows: $O=A_2O<A1<B<AB$ in increasing order.

An alternative explanation for how ABO groups may influence the proteolysis of vWf has been suggested: in individuals with blood group O, the A_2 domain (the site of vWf proteolysis by ADAMTS13) adopts a conformation that is more permissive for ADAMTS 13 cleavage. In contrast, the A and B antigens protect against vWf proteolysis, while vWf purified from individuals with blood group O are cleaved faster by ADAMTS13 protease[43].

Other authors[52], found a correlation between vWf levels and age as we did also. When we compared the behavior of young and older people (with a cut-off point of 50 years) we observed lower levels of vWf in young than in older people, with a mean difference of 35%. Both ages followed the same order of levels depending on the *ABO* genotype described in our population.

Our results showed that vWf distribution relates to *ABO* genotype are independent of sex and individual group (case-control).
In conclusion, low levels of ADAMTS13 behave as an independent risk factor for venous thrombosis in Spanish women. Also vWf levels are blood group dependent while ADAMTS13 levels are not. We observed that *A_2O* genotype had a similar behavior to the *O* genotype only with vWf. The order of vWf levels depending on *ABO* genotype in our population was $O=A_2O<A1<B<AB$. Finally,

the vWf distribution depending on ABO group is independent of sex and individual group (case-control).

Further studies are necessary to support these results.

Limitations of the research:

The size of our sample (214 patients and 224 controls) could have provided less statistical power. Further studies with a larger population are necessary to support our results.

Acknowledgements

We would like to thank Professor Bill Stone and Dr. Montserrat Borrell for reviewing the manuscript.

Sources of funding

Spanish grants: RIC, RD12/0042/0032

Conflict of interest:

None.

REFERENCES:

1. Vischer UM. Von Willebrand factor, endothelial dysfunction, and cardiovascular disease. J *Thromb Haemost 2006; 4: 1186-1193*
2. Whincup PH, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, Rumbley A, Lowe GD. Von Willebrand factor and coronary heart disease: prospective study and meta-analysis. *Eur Haert J 2002; 23: 1764-70*
3. Danesh J, Wheeler JG, Hirscfield GM, Eda S, Eiriksdottic G, Rumley A, Lowe GD, Pepys MB, Gudnason V. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Eng J Med 2004; 350: 1387-97*
4. Folsom AR, Rosamond WD, Sjahar E, Cooper LS, Aleksic N, Nieto FJ, Rasmunssen ML, Wu KK. Prospective study of markers of hemostatic

function with risk of ischemic stroke. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Circulation 1999 17;100(7):736-42.*
5. Preston AE, Barr A. The plasma concentration of factor VIII in the normal population. Br J Haematol 1964;10:238-245
6. Orstavik K, Magnus P, Reisner H, et al. Factor VIII and factor IX in a twin population: evidence for a major effect of ABO locus on factor VIII level. Am J Hum Genet 1985; 37:89-101
7. Gill JC, Endres-Brooks J, Bauer PJ, et al. The effect of ABO blood group on the diagnosis of von Willebrand disease. Blood 1987;69:1691-1695
8. Tirado I, Mateo J, Soria JM, Oliver A, Martínez-Sánchez E, Vallvé C, Borrell M, Urrutia T, Fontcuberta J. The ABO blood group genotype and factor VIII levels as independent risk for venous thromboembolism. Thromb Haemost 2005; 93:468-74
9. Jenkins PV, O'Donell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all?. Transfusion 2006;46:1836-1844
10. O'Donell J, Boulton FE, Manning RA, et al. Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. Arterioscler Thromb Vasc Biol 2002;22:335-341
11. Shima M, Fujimura Y, Nishiyama T, et al. ABO blood group genotype and plasma von Willebrand factor in normal individuals. Vox Sang 1995;68:236-240
12. SoutoJC, Almasy L, Muniz-Díaz E, et al. Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. Arterioscler Thromb Vasc Biol 2000;20:2024-2028
13. Schleef M, Strobel E, Dick A, et al. Relationship between ABO and secretor genotype with plasma levels of factor VIII and von Willebrand factor in thrombosis patients and controls individuals. Br J Haematol 2005;128:100-107
14. Lowe J. The blood group-specific human glycosyltransferases. Baillieres Clin Haematol. 1993;6:465-490
15. Yamamoto F, McNeil PD, Hakomori S. Human histo-blood group A2 transferase coded by A2 allele, one of the A subtypes, is characterized by a single base deletion in the coding sequence, which results in an

additional domain at the carboxyl terminal. Biochem Biophys Res Commum 1992;187:366-374

16. Chauhan AK, Kisucka J, Brill A et al. ADAMTS 13: a new link between thrombosis and inflammation. *J Exp Med 2008;205:2065-2074.*
17. Moake JL, Rudy CK, Troll JH, et al Unusually large plasma factor VIII:von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med 1982;307:1432-1435*
18. Furlan M, Robles R, Solenthaler M et al. Deficient activity of von Willebrand factor-cleving protease in chronic relapsing thrombotic thrombocytopenic purpura. *Blood 1997;89:3097-3103.*
19. Andersson HM, Siegerink B, Luken BM, Crawley JT, Algra A, Lane DA, Rosendaal FR. High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. *Blood 2012; 119: 1555-60.*
20. Matsukawa M, Kaikitia K, Soejima K, Fuchigami S, Nakamura Y, Honda T, Tsujita K, nagayoshi Y, Kojima S, Shimomura H, Sugiyama S, Fujimoto K, Yoshimura M, Nakagaki T, Ogawa H. Serial changes in von Willebrand factor-cleaving protease (ADAMTS 13) and prognosis after acute myocardial infarction. *Am J Cardiol 2007; 100: 758-63*.
21. Kaikita K, Soejima K, Matsukawa M, Nakagaki T, Ogawa H. Reduced von Willebrand factor-cleaving protease (ADAMTS 13) activity in acute myocardial infarction. *J Thromb Haemost 2006;4: 2490-3.*
22. Crawley JT, Lane DA, Woodward M, Rumley A, Lowe GD. Evidence that high von Willebrand factor and low ADAMTS-13 levels independently increase the risk of a non-fatal attack. *J Thromb Haemost 2008; 6: 583-8*.
23. Bongers TN, de Bruijne EL, Dippel DW, de Jong AJ, Deckers JW, Poldermans D, de Maat MP, Leebeck FW. Lower levels of ADAMTS13 are associated with cardiovascular disease in young patients. *Atherosclerosis 2009; 207: 250-4.*
24. Fujioka M, Nakano T, Hayakawa K, Irie K, Akitake Y, Sakamoto Y, Mishima K, Muroi C, Yonekawa Y, Banno F, Kokame K, Miyata T, Nishio K, Okuchi K, Iwasaki K, Fujiwara M, Siesjo BK. ADAMTS 13 gene deletion enhances plasma high-mobility group box 1 elevation and neuroinflammation in brain ischemia-reperfusion injury. *Neurol Sci 2012;33:1107-15*

25. Zhao BQ, Chauhan AK, Canault M, Patten IS, Yang JJ, Dockal M, Scheifinger F, Wagner DD. Von Willebrand factor-cleaving protease ADAMTS 13 reduces ischemic brain injury in experimental stroke. *Blood 2009;114:3329-34.*
26. Bongers TN, De Maat MP, van Goor ML, Bhagwanbali V, van Vliet HH, Gomez Garcia AB, Dippel DW, Leebeek FW. High von Willebrand factor levels increase the risk of first ischemic stroke: influence of ADAMTS 13 inflammation, and genetic variability. *Stroke 2006;37:2672-7.*
27. Stepanian A, Cohen-Moatti M, Sangliar T, Legendre P, Ameziane N, Tsatsaris V, Mandelbrot L, De PD, Veyradier A. von Willebrand factor and ADAMTS 13: a candidate couple for preeclampsia pathophysiology. *Arterioscler Thromb Vasc Biol 2011:31:1703-9.*
28. Aref S, Goda H. Increased VWF antigen levels and decreased ADAMTS 13 activity in preeclampsia.*Hematology 2013;18(4):237-41*.
29. de Mast Q, Groot E, Asih PB, Syafruddin D, Oosting M, Sebastian S, Ferwerda B, Netea MG, de Groot PG, van der Ven AJ, Fijmheer R. ADAMTS deficiency with elevated levels of ultra-large and active von Willebrand factor in *P. falciparum* and *P.vivax* malaria. *Am J Trop Med Hyg 2009;80:492-8.*
30. Larkin D, de LB, Jenkins PV, Bunn J, Craig AG, Terraube V, Preston RJ, Donkor C, Grau GE, van Mourik JA, O'Donell JS. Severe *plasmodium falciparum* malaria is associated with circulating ultra-large von Willebrand multimers and ADAMTS 13 inhibition. *P LoS Pathog 2009;5:e1000349.*
31. Bridges DJ, Bunn J, van Mourik JA, Grau G, Preston RJ, Molyneux M, Combes V, O'Donnell JS, De LB, Craig A. Rapid activation of endothelial cells enables *Plasmodium falciparum* adhesion to platelet-decorated von Willebrand factor strings. *Blood 2010; 115:1472-4.*
32. Lowenberg EC, Charunwatthana P, Cohen S, van den Born BJ, Meiers JC, Yunnus EB, Hassan MU, Hoque G, Maude RJ, Nuchsongsin F, Levi M, Dondorp AM. Severe malaria is associated with a deficiency of von Willebrand factor cleaving protease, ADAMTS13. *Thromb Haemost 2010; 103:181-7.*
33. Kraisin S, Naka I, Patarapotikul J, Nantakomol D, Nuchnoi P, Hananantachai H, Tsuchiya N, Ohashi J. Association of ADAMTS 13 polymorphism with cerebral malaria. *Malar J 2011; 10:366*

34. Michelle A.H. Sonneveld, Moniek P.M. de Maat, Frank W.G. Leebeek. Von Willebrand factor and ADAMTS13 in arterial thrombosis: a systematic review and meta-analysis. *Blood Reviews 2014;28:167-178.*
35. Tsai AW, Cushman M, Rosamond WD, et al. Coagulation factors, inflammation markers, and venous thromboembolism: the longitudinal investigation of thromboembolism etiology (LITE). *Am J Med.2002; 113(8):636-642*.
36. Lijfering Wm, Rosendaal FR, Cannegieter SC. Risk factors for venous thrombosis- current understanding from an epidemiological point of view. *Br J Haematol. 2010; 149(6):824-833*.
37. Smith NL, Rice KM, Bovill EG, et al. Genetic variation associated with plasma von Willebrand factor levels and the risk of incident venous thrombosis. *Blood 2011; 117(22):6007-6011.*
38. Souto JC,, Almasy L, Borrell M, Blanco-Vaca F, Mateo J, Soria JM, Coll I, Felices R, Stone W, Fontcuberta J, Blangero J. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. *Am. J. Hum. Genet. 2000; 67:1452-1459.*
39. Mazetto B.M, Orsi F.L, Barnabé A, De Paula E. V, Flores-Nascimento M.C, Annichino-Bizzacchi J.M. Increased ADAMTS13 activity in patients with venous thromboembolism. *Thromb Res 2012; 130: 889-893*.
40. Maino A, Siegerink B, Lotta L.A., Crawley J.T.B., Le Cessie S., Leebeek F.W.G., Lane D.A., Lowe G.D.O., Peyvandi F, Rosendaal F.R. Plasma ADAMTS-13 levels and the risk of myocardial infarction: an individual data meta-analysis. *J Thromb Haemost. 2015; 13:1396-1404.*
41. Chion CK, Doogen CJ, Crawley JT, Lane DA, Roseendal FR. ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men. *Blood 2007; 109:1998-2000.*
42. Peyvandi F, Hollestelle MJ, Palla R, Merlini PA, Feys HB, Vanhoorelbeke K, Lenting PJ, Mannucci PM. Active platelet-binding conformation of plasma von Willebrand factor in young women with acute myocardial infarction. *J Thromb Haemost 2010; 8: 1653-6*.
43. Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS 13. J Thromb Haemost 2003;1:33-40.
44. McKinnon TA, et al. N-linked glycosylation of VWF modulates its interaction with ADAMTS 13. Blood 2008; 111:3042-3049

45. McGrath RT, et al. Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS 13 proteolysis. Blood 2013;122:4107-4110.
46. McGrath RT, et al. Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS 13. Blood 2010;115:2666-2673
47. Tirado I, Soria JM, Mateo J, Oliver A, Souto JC, Santamaria A, et al. Association after linkage analysis indicates that homozygosity for the 46C>T polymorphism in the F 12 gene is a genetic risk factor for venous thrombosis. Thromb Haemost 2004;91:899-904
48. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res 1988;16:1215.
49. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, et al. Heart disease and stroke statistics-2014 update: a report from American Heart Association. *Circulation 2014; 129: e28-292*.
50. Morrelli VM, de Visser MCH, van Tilburg NH, Vos HL, Eikenboom JCJ, Rosendaal FR, Bertina RM. ABO blood group genotypes, plasma von Willebrand factor levels and loading of von Willebrand factor with A and B antigens. Thromb Haemost 2007; 97:534-541.
51. Anwar M. Al-Awadhi, Seham K. Al-Sharrah, Mehrez M. Jadaon, Faisal Al-Sayegh. Investigating the influence of age, gender and ABO blood group on ADAMTS-13 antigen and activity levels in healthy Arabs. Blood Transfus 2014: 12: 138-40
52. Emmanuel J. Favaloro, PhD; Soma Soltani; Jane McDonald; Ella Grezchnik; Leanne Easton; James W.C. Favaloro. Reassessment of ABO Blood Group, Sex, and Age on Laboratory Parameters Used to Diagnose von Willebrand Disorder. Potential Influence on the Diagnosis vs the Potential Association With Risk of Thrombosis. Am J Clin Pathol. 2005; 124(6):910-917.

ANNEX 2 CAPÍTOL 4.3

En aquest annex parlarem dels resultats obtinguts en la població RETROVE. Els resultats obtinguts en el nostre estudi (capítol 4.3) no es van replicar en la població RETROVE, vam obtenir les següents resultats:

Percentil 10 d'ADAMTS13

		>0,59 (µg/mL)	≤0,59 (µg/mL)	OR (CI 95%)	OR ajustada per edat	TOTAL
TOTS	Control	356 (89%)	44 (11%)	1,69 (1,13-2,55) p=0,012	0,94 (0,60-1,47) NS	400
	Pacient	331 (82,8)	69 (17,3%)			400
Dones	Control	187 (90,8%)	19 (9,2%)*	1,83 (1,01-3,40) p=0,049	1,15 (0,59-2,26) NS	206
	Pacient	172 (84,3%)	32 (15,7%)*			204
Homes	Control	169 (87,1%)	25 (12,9%)	1,57 (0,91-2,76) NS	0,76 (0,40-1,42) NS	194
	Pacient	159 (81,2%)	37 (18,8%)			196

Si bé vam trobar diferències significatives entre pacients i controls en les dones amb nivells baixos d'ADAMTS13 al ajustar per edat les diferències significatives es van perdre.
Es va calcular el risc trombòtic del FvW en la població RETROVE:

Percentil 90 de vWf

		FvW (<183%)	FvW (≥183%)	OR (CI 95%)	OR ajustada per edat	TOTAL
TOTS	Controls	359(89,8%)	41 (10,2%)*	11,32 (7,75-16,54) p=0,0001	8,48 (5,48-13,12) p= 0,0001	400
	Pacients	174(43,6%)	225(56,4%)*			399

Vam calcular també la OR de la combinació d'ADAMTS13 baixos i FvW alts en la població RETROVE, vam obtenir els següents resultats:

FvW-ADAMTS13

		FvW(<183%)	FvW (≥183%)	OR (95%CI)	OR ajustada per edat	TOTAL
ADAMTS13 ≤0,59 (µg/mL)	Controls	35 (79.5%)	**9 (20.5%)***	14 (5.75-37.31) p=2.62e-08	13.79 (5.40-9.07) p=1.62e-07	44
ADAMTS13 ≤0,59 (µg/mL)	Pacients	15 (21.7%)	**54 (78.3%)***			69

Obtenim una OR molt alta quan combinem els nivells d'ADAMTS13 amb els del FvW. Observem que en els individus amb nivells baixos d'ADAMTS13 combinats amb nivells alts de FvW hi ha sis vegades més pacients que controls (54 vs 9). Aquestes diferències significatives es van mantenir quan vam ajustar per l'edat.

L'associació entre els nivells alts de FvW i el risc trombòtic augmenta notablement quan afegim els nivells disminuïts d'ADAMTS13 a l'anàlisi (OR=8.48 i OR= 13.79 respectivament)

Observem també que els individus amb ADAMTS13 baixos (<0.69) tenen FvW més alts (Figura 1) i són més vells (Figura 2):

Figura 1:

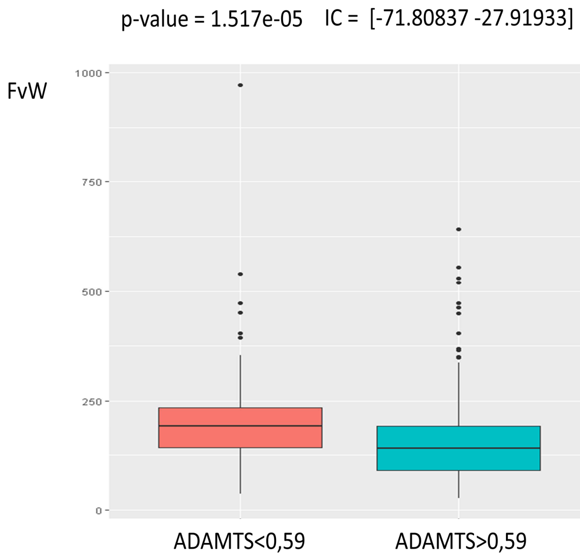
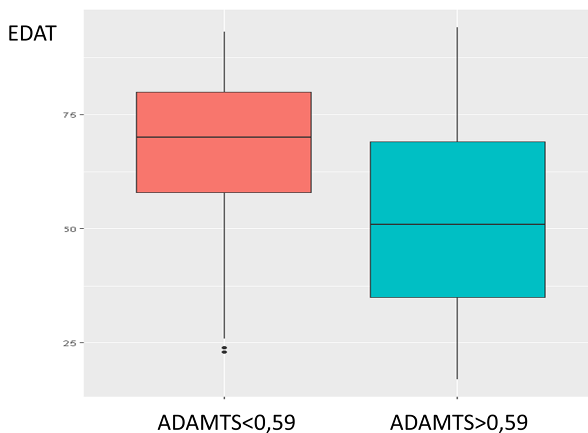


Figura 2:



Veiem doncs que els nivells d'ADAMTS13 disminueixen amb l'edat i els del FvW augmenten. Obtenim les següents correlacions:

Correlació dels nivells d' ADAMTS13 amb l'edat $R= -0.291$ ($p< 2.2e-16$) i correlació dels nivells de FvW amb l'edat $R= 0.4795$ ($p< 2.2e-16$).

CAPÍTOL 4.4

INFLUENCE OF ABO LOCUS ON PFA-100 COLLAGEN-ADP CLOSURE TIME IS NOT TOTALLY DEPENDENT ON THE VON WILLEBRAND FACTOR. RESULTS OF A GWAS ON GAIT-2 PROJECT PHENOTYPES

CAPÍTOL 4.4

INFLUENCE OF ABO LOCUS ON PFA-100 COLLAGEN-ADP CLOSURE TIME IS NOT TOTALLY DEPENDENT ON THE VON WILLEBRAND FACTOR. RESULTS OF A GWAS ON GAIT-2 PROJECT PHENOTYPES

RESUM:

Introducció: En estudis previs, hem trobat que dos fenotips relacionats amb la reactivitat plaquetària, mesurada amb el sistema PFA-100, són altament heretables. L'objectiu del estudi present va ser identificar determinants genètics que influeixen en la variabilitat d'aquests fenotips: temps d'oclusió del col·lagen_ADP (Col-ADP) i del col·lagen-epinefrina (Col-Epi).

Mètodes: Com a part del GAIT -2 (projecte de Genetic Analysis of Idiopathic Thrombophilia-2), es van estudiar 935 individus de 35 famílies grans espanyoles. Es va realitzar un estudi d'associació del genoma complet (GWAS) amb ≈ 10M de polimorfismes de nucleòtid únic (SNPs) amb els fenotips Col-ADP i Col-Epi.

Resultats: L'estudi va donar senyals genètiques significants que mapejaven el locus *ABO*. Després d'ajustar els dos fenotips pel genotip *ABO*, aquestes senyals desapareixien. Després d'ajustar per factor von Willebrand (VWF) o pel factor VIII de coagulació (FVIII), les senyals significatives desapareixien totalment pel fenotip Col-Epi però només parcialment pel fenotip Col-ADP.

Conclusions: Els nostres resultats suggereixen que el locus *ABO* exerceix la principal influència genètica en els fenotips de PFA-100. Tanmateix, mentre l'efecte del locus *ABO* sobre el fenotip Col-Epi es mediat totalment pel VWF i/o FVIII, l'efecte del locus *ABO* sobre el fenotip Col-ADP es parcialment produït pel VWF i/o FVIII, i parcialment per altres mecanismes.

International Journal of
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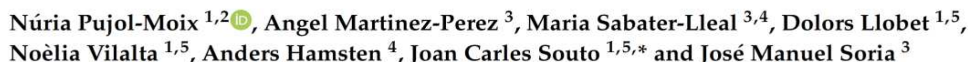
Influence of *ABO* Locus on PFA-100 Collagen-ADP Closure Time Is Not Totally Dependent on the Von Willebrand Factor. Results of a GWAS on GAIT-2 Project Phenotypes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Received: 6 June 2019; Accepted: 27 June 2019; Published: 30 June 2019

Abstract: (1) Background: In a previous study, we found that two phenotypes related to platelet reactivity, measured with the PFA-100 system, were highly heritable. The aim of the present study was to identify genetic determinants that influence the variability of these phenotypes: closure time of collagen-ADP (Col-ADP) and of collagen-epinephrine (Col-Epi). (2) Methods: As part of the GAIT-2 (Genetic Analysis of Idiopathic Thrombophilia (2) Project, 935 individuals from 35 large Spanish families were studied. A genome-wide association study (GWAS) with ≈ 10 M single nucleotide polymorphisms (SNPs) was carried out with Col-ADP and Col-Epi phenotypes. (3) Results: The study yielded significant genetic signals that mapped to the *ABO* locus. After adjusting both phenotypes for the *ABO* genotype, these signals disappeared. After adjusting for von Willebrand factor (VWF) or for coagulation factor VIII (FVIII), the significant signals disappeared totally for Col-Epi phenotype but only partially for Col-ADP phenotype. (4) Conclusion: Our results suggest that the *ABO* locus exerts the main genetic influence on PFA-100 phenotypes. However, while the effect of the *ABO* locus on Col-Epi phenotype is mediated through VWF and/or FVIII, the effect of the *ABO* locus on Col-ADP phenotype is partly produced through VWF and/or FVIII, and partly through other mechanisms.

Keywords: platelet reactivity; platelet function test; *ABO* blood-group system; von Willebrand factor; factor VIII

1. Introduction

Platelet reactivity can be measured using a wide variety of laboratory functional tests. Such tests are classified into two main types [1]: (1) tests based on platelet aggregation activated by agonists, in platelet-rich plasma or in whole blood; (2) tests based on platelet adhesion under shear stress. Among the second group of tests, the PFA-100 system (Siemens Healthcare Diagnostics, Marburg, Germany) measures platelet function by simulating in vitro a vessel wall under shear stress. The vessel wall is simulated by a membrane coated with collagen; it is coated also with ADP (cartridge collagen-ADP) or with epinephrine (cartridge collagen-epinephrine) as platelet agonists. The membrane has a hole through which the anticoagulated blood passes; the closure times (CTs) of this hole are inversely

proportional to the functional capacity of platelets. The PFA-100 system was introduced in 1995 with the objective of measuring primary hemostasis in whole blood in vitro as a non-invasive and more accurate method than the bleeding time in vivo [2,3]. Long CTs gave reliable measurements of hemostatic deficiencies due to low levels of von Willebrand factor (VWF) or platelet functional defects [2,4]. The use of PFA-100 was subsequently expanded to the assessment of the effect of platelet hyperreactivity, as indicated by shortened CTs, on arterial and venous thrombotic risk [3,5–8]. Citrate concentration, platelet count, hematocrit, white blood cell count, circadian rhythm and some dietary elements can influence the CTs [3,9,10]. Age and sex do not have appreciable influence although slight shortening of CTs has been described in neonates, in children and in elderly men [3,10,11]. Obviously, VWF has an important influence on CTs, which correlates inversely with VWF levels [3,4,9,10]. Also, the *ABO* blood group influences PFA-100 CTs, with O group individuals having longer CTs than those of non-O groups [3,4,9,10,12,13]; this has been interpreted as an effect of the lower levels of VWF in group O individuals [4,9,10]. Some authors have suggested that factor VIII (FVIII) has no impact on the PFA-100 CTs [2,5], while others have found the contrary [14].

The GAIT-2 (Genetic Analysis of Idiopathic Thrombophilia (2) Project is a family-based genetic study designed to identify new genetic markers of thrombotic risk [15]. Using the variance component statistical method, the heritability of intermediate phenotypes that could play a role in thrombotic risk was determined. In the GAIT-2 Project, two PFA-100 phenotypes were included as a measure of platelet reactivity: CTs of collagen-ADP cartridge (Col-ADP) and of collagen-epinephrine cartridge (Col-Epi). Both phenotypes showed a strong genetic component with a heritability of 0.45 for Col-ADP and 0.52 for Col-Epi [16].

The objectives of the present study were: (1) to analyze the genetic correlations between Col-ADP and Col-Epi phenotypes with each other and with other related phenotypes, and (2) to perform a genome-wide association study (GWAS) to identify susceptibility loci for Col-ADP and Col-Epi phenotypes.

2. Results and Discussion

2.1. Genetic Correlations of the PFA-100 Phenotypes

The genetic correlations of Col-ADP and Col-Epi with each other and with the VWF antigen, coagulant FVIII and *ABO* genotype (considering dominant effect of allele *O*) are presented in Table 1.

Table 1. Genetic correlations among PFA phenotypes, von Willebrand factor, coagulation factor VIII and *ABO* genotype.

Phenotype	Col-Epi	VWF	FVIII	*ABO*
Col-ADP	$\rho = 0.7917$ (5.80×10^{-9})	$\rho = -0.7002$ (1.02×10^{-10})	$\rho = -0.6209$ (2.66×10^{-8})	$\rho = 0.5895$ (7.01×10^{-9})
Col-Epi	-	$\rho = -0.6342$ (7.14×10^{-8})	$\rho = -0.5947$ (3.97×10^{-7})	$\rho = 0.4477$ (0.0003)

ρ = genetic correlation and *p*-value (in brackets); VWF = von Willebrand factor antigen; FVIII = coagulation factor VIII activity; *ABO* = *ABO* genotype, considering dominant effect of allele *O*.

As expected, the genetic correlations of PFA-100 phenotypes with VWF and *ABO* blood group were statistically significant. In addition, a significant correlation between the PFA-phenotypes and FVIII was also observed. Unlike VWF, which has an important role in primary hemostasis, FVIII is fundamental for coagulation. The mechanism by which the FVIII influences the PFA-100 CTs could be partially explained by its close relationship with the VWF. Both factors circulate together, and their levels are related. Moreover, genetic studies have demonstrated that there is a huge overlap between genetic factors regulating FVIII and VWF [17,18].

2.2. GWAS of the PFA-100 Phenotypes

Manhattan plots of the GWAS on Col-ADP and Col-Epi phenotypes are shown in Figure 1.

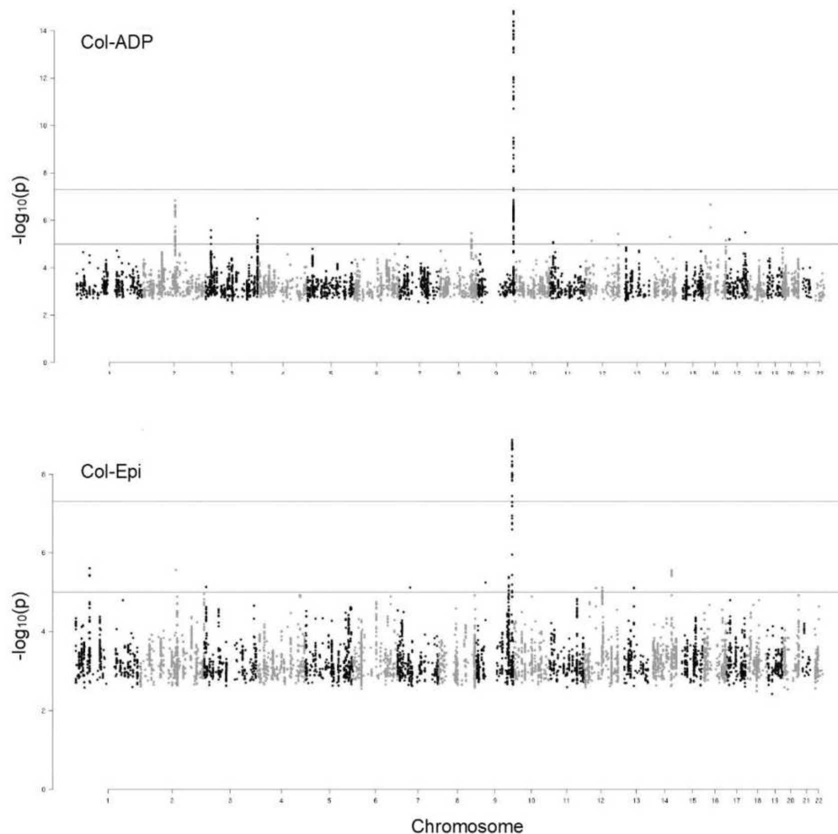

Figure 1. Manhattan plots of the GWAS on two PFA-100 phenotypes: collagen-ADP and collagen epinephrine closure times. Dots correspond to SNPs organized by chromosomal order and position and the vertical axis shows the statistical significance expressed as $-\log_{10}$ of the p-values. The horizontal lines mark the 5×10^{-8} p-value threshold of genome-wide significance.

For each phenotype, a signal on chromosome 9 at the *ABO* locus reached a genome-wide significance level (p-value $< 5 \times 10^{-8}$). Seventy single nucleotide polymorphisms (SNPs) were found to be associated with Col-ADP, with p-values up to 1.50×10^{-15}. Of these 70 SNPs, 45 were associated also with Col-Epi but with less statistical significance: p-values up to 1.35×10^{-9}. The complete list of these SNPs is described in Supplementary Table S1. For both Col-ADP and Col-Epi phenotypes, all significant SNPs were located within the *ABO* gene and in its adjacent intergenic region.

To the best of our knowledge, our study is the first GWAS of PFA-100 phenotypes. Previous GWAS reports of platelet reactivity phenotypes were mainly based on aggregometry. Using GWAS and other genetic approaches, several SNPs have been found in association with platelet aggregometry phenotypes. These were located in different genes related to platelet functional receptors, regulators of cytoskeleton and signaling proteins. Among these genes, the following were described: *MIR100HG*, *MME*, *PIP3-E*, *GLIS3*, *LDHAL6A*, *ANKS1B*, *PIK3CG*, *MAGI1*, *C8orf86*, *FGFR1*, *LPAR1*, *CACNB2*, *SLC39A12*, *RPP25*, *SCAMP5*, *BMPR1A* (revisited in Bunimov et al. [19]), *ANKRD26* [20], *pannexin* [21], *ADRA2* [22] and the most relevant *PEAR-1* [22–24]. Notably, none of these genetic variants was found in the *ABO* locus. On the other hand, in our study, we did not find any of the SNPs previously reported to be associated with platelet aggregometry. To explain these different results, we should keep in mind that aggregometry, both in platelet-rich plasma and in whole blood, analyzes aggregation, that is, the platelet-platelet binding mediated by fibrinogen. In contrast, the PFA-100 test basically analyzes adhesion under shear stress, which depends mainly on binding with VWF [2,10].

Nineteen of the SNPs that we found in the *ABO* locus have been previously reported in relation to thrombotic-related conditions (Table 2): Venous thromboembolism [25–30], myocardial infarction [31], large-vessel and cardioembolic stroke [32], large-artery arteriosclerosis [33], coronary artery disease [34,35] and coronary artery disease shared with venous thromboembolism [36]. The association of these SNPs with thrombosis and with the PFA-100 phenotypes suggests that this functional platelet test may be useful to estimate thrombotic risk, although further studies are needed to confirm this. Moreover, some of the SNPs have been described also in association with variations in VWF, FVIII, and/or variations in biological factors that may play indirect roles in thrombosis, such as adhesion molecules [17,18,37–39].

Table 2. *ABO* locus (chromosome 9): SNPs associated with PFA-100 phenotypes which have been previously described in association with thrombosis-related conditions and with variations of biological factors.

SNP	Position (bp)	Location	MAF	Association with Col-ADP *p*-Value	Association with Col-Epi *p*-Value	Association with Thrombosis-Related Conditions [References]	Association with Variations of Biological Factors [References]
rs8176719	136132908	coding, 5-UTR, intron	0.459	5.21×10^{-14}	1.88×10^{-9}	VTE [18,25,26,29]	
rs687621	136137065	intron	0.432	1.49×10^{-15}	3.50×10^{-9}	VTE [18,26], MI [31], LVCES [32]	VWF [17,18,38], ICAM-1 [38]
rs687289	136137106	intron	0.433	1.93×10^{-15}	5.72×10^{-9}	MI [31], LVCES [32]	FVIII [16,17], ICAM-1 [38]
rs2519093	136141870	intron	0.312	1.16×10^{-12}	6.01×10^{-9}	VTE [25], LVCES [32]	-
rs514659	136142203	intron	0.433	6.04×10^{-15}	1.04×10^{-8}	VTE [26], MI [31], LVCES [32], LAA [33]	VWF [38]
rs644234	136142217	intron	0.460	1.42×10^{-14}	2.18×10^{-9}	MI [31], LVCES [32]	E-selectin [38]
rs643434	136142355	intron	0.460	1.43×10^{-14}	2.18×10^{-9}	MI [31], LVCES [32]	-
rs545971	136143372	Intron	0.433	6.03×10^{-15}	1.04×10^{-8}	MI [31], LVCES [32]	-
rs612169	136143442	intron	0.433	6.05×10^{-15}	1.04×10^{-8}	MI [31], LVCES [32]	ICAM-1, E-selectin [38]
rs674302	136146664	intron	0.433	6.06×10^{-15}	1.04×10^{-8}	MI [31], LVCES [32]	-
rs500498	136148647	intron	0.408	5.56×10^{-08}	-	VTE [27], LVCES [32]	ICAM-1, E-selectin [38]
rs505922	136149229	intron	0.433	9.68×10^{-15}	1.12×10^{-8}	VTE [27,28], MI [31], LVCES [32]	-
rs529565	136149500	intron	0.434	1.86×10^{-14}	1.01×10^{-8}	VTE [29,30], MI [31], LVCES [32], LAA [33]	-
rs630014	136149722	intron	0.407	8.74×10^{-10}	-	VTE [27,28], LVCES [32]	E-selectin [38]
rs651007	136153875	intergenic	0.204	8.02×10^{-12}	2.32×10^{-9}	LVCES [32], LAA [33], CAD [34]	VWF, ICAM-1, E-selectin, cholesterol [38]
rs579459	136154168	intergenic	0.340	3.75×10^{-12}	1.35×10^{-9}	LVCES [32], CAD [35], CAD+VTE [36]	ICAM-1, E- and P-selectin [38]
rs649129	136154304	intergenic	0.338	6.88×10^{-12}	2.19×10^{-9}	LVCES [32]	ICAM-1, LDL-cholesterol [38,39]
rs495828	136154867	intergenic	0.340	7.71×10^{-12}	2.28×10^{-9}	VTE [25,27], LVCES [32]	ACE [38]
rs633862	136155444	intergenic	0.391	2.41×10^{-9}	-	LVCES [32]	-

VTE = venous thromboembolism; MI = myocardial infarction; LVCES = large-vessel and cardioembolic stroke; LAA = large-artery arteriosclerosis; CAD = coronary artery disease; CAD + VTE = coronary artery disease shared with venous thromboembolism; VWF = von Willebrand factor; FVIII = coagulant factor VIII; ACE = angiotensin-converting-enzyme.

After finding a genetic correlation between the PFA-100 phenotypes and VWF, FVIII and *ABO* genotype, we adjusted the GWAS results for these factors. As shown in Figure 2 and Supplementary Table S1, after adjusting the Col-ADP results for VWF 50, SNPs remained significant but with lower significance than before adjustment (*p*-values up to 1.55×10^{-10}). After adjusting the Col-ADP, the results for FVIII 46 SNPs remained significant; almost all were the same as for the VWF adjustment, but 6 were different. The remaining SNPs also had a lower significance than before (*p*-values up to 5.00×10^{-11}).

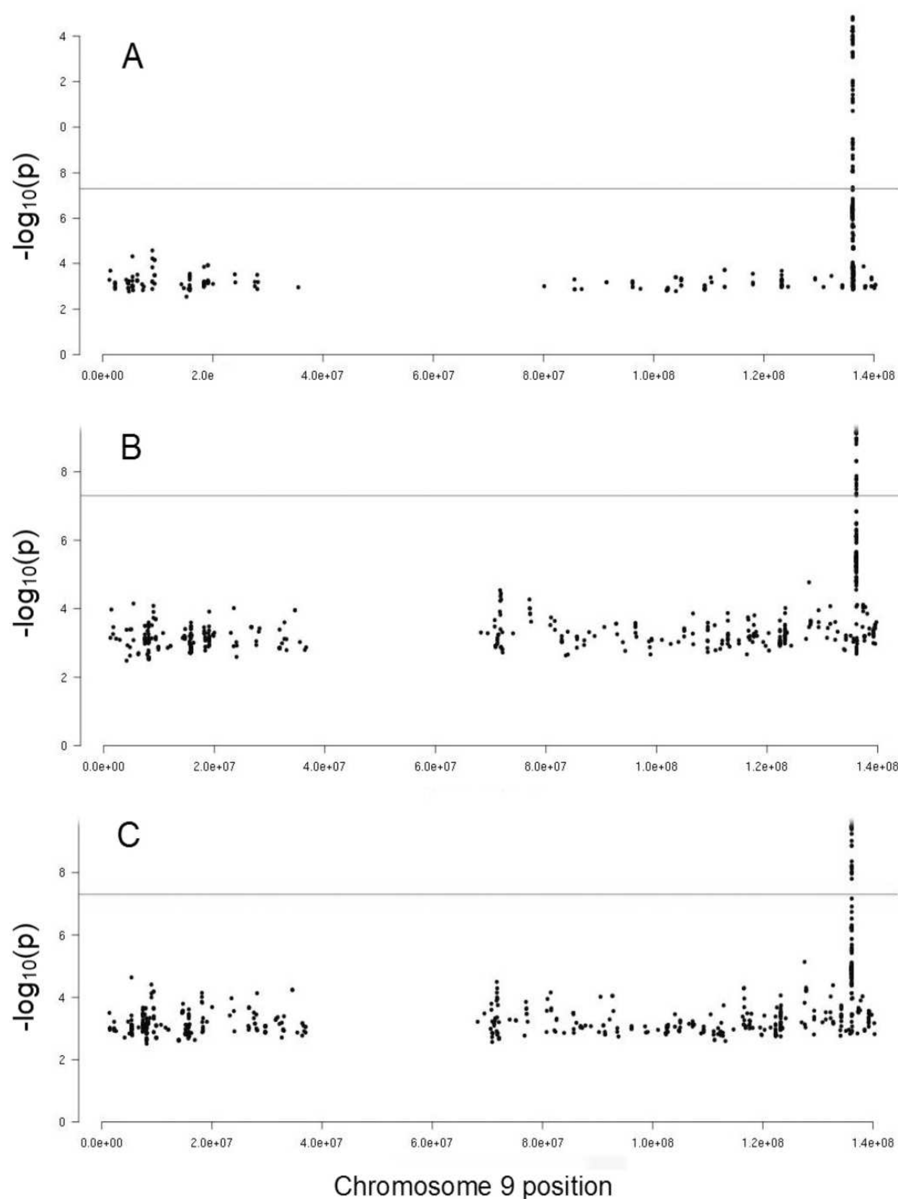

Figure 2. Manhattan plots of the chromosome 9 region of the GWAS on collagen-ADP closure time phenotype: (**A**) without adjustments, (**B**) after adjusting for von Willebrand factor, and (**C**) after adjusting for factor VIII. Dots correspond to SNPs organized by position and the vertical axis shows the statistical significance expressed as -$\log_{10}$ of the *p*-values. The horizontal lines mark the 5×10^{-8} *p*-value threshold of genome-wide significance.

It should be noted that after adjustment of Col-ADP results for *ABO* genotype, the association signal disappeared completely (data not shown). Regarding Col-Epi, the significant SNPs disappeared with any of the adjustments performed, including VWF, FVIII and *ABO* genotype.

2.3. Genetic Influence of ABO Locus on PFA-100 Phenotypes

Our GWAS results and adjustments suggested that the *ABO* gene was the main determinant of variations in PFA-100 CTs since all the significant SNPs associated with PFA-100 phenotypes were at the *ABO* locus and all of them lost their genome-wide significance when we adjusted for the *ABO* genotype.

However, there were differences between Col-ADP and Col-Epi phenotypes. With Col-Epi, all significant SNPs also disappeared when adjusted for VWF or FVIII. This suggested that the influence of the *ABO* gene was related mainly to these factors. Regarding Col-ADP, the adjustment either by VWF or by FVIII reduced the number of significant SNPs in both cases but 50 and 46 respectively did not disappear. This suggested that part of the genetic effect of the *ABO* gene on the Col-ADP was mediated by VWF and/or FVIII. The relationships among *ABO* blood group, VWF and FVIII have been previously described. Non-O group individuals have 25% higher levels of VWF and FVIII than O group individuals [37,38]. These differences are attributed to the formation of A and B antigens, catalyzed by specific glucosyltransferases, on the H antigen existing in VWF. They are related to the glycosylation and clearance rate of VWF which is lower in non-O individuals. We previously showed that FVIII and VWF were genetically correlated with thrombotic risk, and demonstrated significant linkage between the *ABO* locus and plasma levels of VWF and FVIII [40]. More recent GWAS demonstrated that *ABO* gene is by far the major determinant of VWF and FVIII levels [18]. This can explain the genetic relationship between the PFA-100 phenotypes and the *ABO* blood group through the VWF and/or FVIII.

According to our results, part of the effect of the *ABO* locus on the Col-ADP phenotype does not occur due to WVF and/or FVIII. Some studies have suggested alternative mechanisms by which the *ABO* gene can intervene in platelet reactivity, thrombosis, and cardiovascular diseases. Non-O individuals have higher levels of cholesterol [41]. Also, they have increased amounts of the adhesion molecules and cytokines involved in inflammation that are related to cardiovascular disease [38,39,42]. Ten of the SNPs that we found at the *ABO* locus, associated with PFA-100 phenotypes, have been described previously in association with adhesion molecules and/or cholesterol (Table 2).

Moreover, the *ABO* blood group can influence platelet reactivity through various mechanisms related to glycosylation [37,43]. A and B antigens expressed on platelet glycoproteins can modify the activity of the glycoprotein complexes involved in platelet adhesion and the galectin-glycan interactions; galectins from extracellular matrices are potent platelet agonists. A and B antigens, also present on platelet glycosphingolipids, are involved in platelet aggregation and thrombosis by binding cell adhesion molecules.

Glycosylation is essential also for other platelet functions in which no relationship with the *ABO* blood group has been described so far. An example of this is the glycosylation of the P2Y12 receptor [44]. P2Y12 is a G_i-coupled ADP receptor that contains two potential N-linked glycosylation sites at its extracellular amino-terminus. The lack of glycosylation of this receptor leads to a defective P2Y12-mediated inhibition of the adenylyl cyclase activity resulting in defective platelet reactivity. The fact that the effect of the *ABO* locus, independent of the VWF and/or FVIII, was demonstrated only for the Col-ADP phenotype gives rise to speculation about the possibility that the *ABO* locus, among other mechanisms, could influence the glycosylation of the P2Y12 receptor. Further data are required to support this hypothesis.

3. Methods

3.1. Enrollment of Individuals and Families

The enrollment of individuals and families was described in detail in a previous publication [16]. Briefly, 935 individuals from 35 large Spanish families, included in the GAIT-2 Project, were recruited through a proband with idiopathic thrombophilia and the condition of having at least of 10 members in at least 3 generations willing to participate in the study. The exclusion criteria were: Deficiencies of antithrombin, protein S, protein C, heparin cofactor II, or plasminogen, activated protein C resistance, Factor V Leiden, dysfibrinogenemia, lupus anticoagulant and antiphospholipid antibodies. The subjects were questioned about their current medication to confirm that they had not taken antiplatelet drugs in the last two weeks, or other drugs with slight effect on platelet function (such as nonsteroidal anti-inflammatory drugs or serotonin reuptake inhibitor drugs) in the last week.

Among the individuals studied, 465 were male and 470 were female. The mean age was 39.5 (minimum 2.6, maximum 101, SD 21.4), and 197 of them were 18-years of age or younger. There were 86 with venous thrombosis, 47 with arterial thrombosis, and 13 with both venous and arterial thrombosis.

The study was performed according to the Declaration of Helsinki. Written informed consent was obtained from all adult patients and from parents or guardians of children. All procedures were approved by the Institutional Review Board at the Hospital de la Santa Creu i Sant Pau. The GAIT-2 Project was approved on November 23, 2005. At that time, no number was assigned to the approved projects.

3.2. Blood Collection, Laboratory Analyses and DNA Preparation

Whole blood samples were obtained by venipuncture, under basal conditions, after a 12 h overnight fast, and between 9:00 a.m. and 9:30 a.m. to minimize the circadian fluctuation. A 5 mL sample was obtained in EDTA-K3 for determining standard blood cell counts. A 5 mL sample of blood was collected in 3.8% sodium citrate to be analyzed in the PFA-100 device; the phenotypes obtained were: CTs (in seconds) for the cartridge collagen-ADP (Col-ADP phenotype) and for the cartridge collagen-epinephrine (Col-Epi phenotype). To avoid erroneous results due to thrombocytopenia or anemia, 6 individuals with platelet counts down to 100×10^9/L and 8 individuals with hemoglobin down to 110 g/L were excluded for the of PFA-100 measurements. Another sample collected in 3.8% sodium citrate was used to obtain platelet-poor plasma by centrifugation at 2000 *g* for 20 min at room temperature. This plasma was used for determining VWF antigen by ELISA and coagulant FVIII activity as previously described [40]. VWF antigen and coagulant FVIII were recorded as percentages of an international standard sample. PFA-100 and FVIII activity assays were performed on fresh samples.

DNA was extracted from EDTA blood samples using a standard salting-out procedure [45] or a commercial kit (Wizard, Promega Corp, Madison, WI, USA). The *ABO* genotype, that distinguishes the A_1, A_2, B, O_1 and O_2 alleles, was determined as previously described [46]. The primers used were described in Souto et al. [40].

3.3. Genotyping Filtering and Imputation

We genotyped the samples from 934 individuals, with a combination of HumanOmniExpressExome-8v1.2 (324 individuals and coverage 964,193 variants) and HumanCoreExome-12v1.1 (610 individuals and coverage 542,585 variants). After filtering the datasets by call rate (>98%), Hardy-Weinberg Equilibrium (p-value > 10^{-6}) and minor allele frequency (MAF) (>1%) and deleting the Mendelian errors, we obtained 395,556 SNPs in all of the samples. We estimated haplotypes using SHAPEIT v2 [47] and imputed genotypes to the 1000 genomes phase 1 panel using IMPUTE2 [48]. We imputed 37,985,264 SNPs, of which 10,844,567 remained after filtering again by MAF (>0.5%). The 14 individuals who had thrombocytopenia or anemia were excluded from the association analysis.

3.4. Statistical Correlation Analyses

The correlations among the phenotypes Col-ADP and Col-Epi with each other and, by pairs, with other biologically-related phenotypes, were analyzed by multivariate variance component models, which are an extension of the univariate model [49]. By studying these traits in extended families, we were able to estimate robustly both the genetic (ρ_g), and the environmental (ρ_e) correlations between pairs of traits.

3.5. GWAS of PFA-100 Phenotypes

We used two phases for the association analysis of normal transformed quantitative phenotypes with the imputed genotypes. The first was a fast screen of variants with Matrix eQTL [50] using modelLINEAR. The GWAS of each phenotype was calculated adjusting by age. Sex, smoking and and oral contraceptives were not included as covariates, since they did not influence the PFA-100 CTs in the GAIT-2 sample [16]. The "errorCovariance" parameter was used to account for the pedigree effect. Variants with a p-value $< 10^{-3}$ were recalculated in the second phase with SOLARIUS [51] and SOLAR-Eclipse v 8.1.1 [52], using variance component methods. Only the p-values calculated with SOLAR are reported, since it is considered a gold standard in the field of extended pedigree samples. SOLAR employs the maximum likelihood approach for variance component models with the standard likelihood ratio tests to evaluate the statistical significance of the model's parameters [53].

4. Conclusions

We report the first GWAS on PFA-100 phenotypes. The results of our study suggest that the *ABO* locus is the main determinant of these phenotypes and, in the case of Col-Epi, all the *ABO* influence is mediated by VWF and/or FVIII. However, for Col-ADP, the influence of *ABO* is only partially mediated by VWF and/or FVIII suggesting that other mechanisms may explain the *ABO* effect on this phenotype. Much remains to be done to better understand the relationships between *ABO* blood group and platelet reactivity measured by PFA-100 test, beyond those mediated by VWF and/or FVIII. Unraveling these mechanisms will help to identify novel pathways involved in platelet reactivity.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/20/13/3221/s1, Table S1. Description of the 72 significant SNPs associated with PFA-100 phenotypes: position in the *ABO* gene (chromosome 9), location, and significance levels. For collagen-ADP only, the same parameters after adjusting for von Willebrand factor and for coagulation factor VIII.

Author Contributions: J.C.S., J.M.S., and N.P.-M. designed and coordinated de study. N.P.-M. and A.M.-P. wrote the article. D.L. and N.V. performed and interpreted the PFA-100 test and performed other laboratory analysis and the *ABO* genotype. A.M.-P. conducted the bioinformatic work related to genotyping and imputation, statistic correlations and GWAS. M.S.-L. and A.H. contributed to data analysis and interpretation of GWAS. All authors critically reviewed and approved the final version of the manuscript.

Funding: This study was supported (in part) by the Spanish Ministerio de Educación y Ciencia, grants SAF 2005-04738 and SAF 2008-08159, and by the Spanish Ministerio de Economía y Competitividad, grants RD12/0042/0032, PI12/00612 and PI15/00269. Maria Sabater-Lleal is a recipient of a Miguel Servet contract from the Spanish Ministerio de Salud – ISCIII, CP17/00142.

Acknowledgments: Genotyping was performed by the SNP&SEQ Technology Platform in Uppsala (www.genotyping.se). The facility is part of the National Genomics Infrastructure supported by the Swedish Research Council for Infrastructures and Science for Life Laboratory, Sweden. The SNP&SEQ Technology Platform is also supported by the Knut and Alice Wallenberg Foundation.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Paniccia, R.; Priora, R.; Liotta, A.A.; Abbate, R. Platelet Function Tests: A Comparative Review. *Vasc Health Risk Manag.* **2015**, *11*, 133–148. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4340464/ (accessed on 17 May 2019). [CrossRef] [PubMed]

2. Harrison, P. The role of PFA-100 testing in the investigation and management of haemostatic defects in children and adults. *Br. J. Haematol.* **2005**, *130*, 3–10. Available online: https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1365-2141.2005.05511.x (accessed on 17 May 2019). [CrossRef] [PubMed]
3. Favaloro, E.J. Clinical utility of the PFA-100. *Semin. Thromb. Hemost.* **2008**, *34*, 709–733. Available online: https://www.thieme-connect.com/products/ejournals/pdf/10.1055/s-0029-1145254.pdf (accessed on 21 June 2019). [CrossRef] [PubMed]
4. Ardillon, L.; Ternisien, C.; Fouassier, M.; Sigaud, M.; Lefrançois, A.; Pacault, M.; Ribeyrol, O.; Fressinaud, E.; Boisseau, P.; Trossaërt, M. Platelet function analyser (PFA-100) results and von Willebrand factor deficiency: A 16-year 'real-world' experience. *Haemophilia* **2015**, *5*, 646–652. Available online: https://onlinelibrary.wiley.com/doi/abs/10.1111/hae.12653 (accessed on 17 May 2019). [CrossRef] [PubMed]
5. Favaloro, E.J. Clinical utility of closure times using the platelet function analyzer-100/200. *Am. J. Hematol.* **2017**, *92*, 398–404. Available online: https://onlinelibrary.wiley.com/doi/full/10.1002/ajh.24620 (accessed on 17 May 2019). [CrossRef] [PubMed]
6. Fuchs, I.; Frossard, M.; Spiel, A.; Riedmüller, E.; Laggner, A.N.; Jilma, B. Platelet function in patients with acute coronary syndrome (ACS) predicts recurrent ACS. *J. Thromb. Haemost.* **2006**, *4*, 2547–2552. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1538-7836.2006.02239.x (accessed on 17 May 2019). [CrossRef] [PubMed]
7. Reny, J.L.; De Moerloose, P.; Dauzat, M.; Fontana, P. Use of the PFA-100 closure time to predict cardiovascular events in aspirin-treated cardiovascular patients: A systematic review and meta-analysis. *J. Thromb. Haemost.* **2008**, *6*, 444–450. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1538-7836.2008.02897.x (accessed on 17 May 2019). [CrossRef]
8. Vázquez-Santiago, M.; Vilalta, N.; Cuevas, B.; Murillo, J.; Llobet, D.; Macho, R.; Pujol-Moix, N.; Carrasco, M.; Mateo, J.; Fontcuberta, J.; et al. Short closure time values in PFA-100® are related to venous thrombotic risk. Results from the RETROVE Study. *Thromb. Res.* **2018**, *169*, 57–63. Available online: https://www.thrombosisresearch.com/article/S0049-3848(18)30420-1/fulltext (accessed on 17 May 2019). [CrossRef]
9. Nazarian, S.M.; Thompson, J.B.; Gluckman, T.J.; Laws, K.; Jani, J.T.; Kickler, T.S.; Rade, J.J. Clinical and laboratory factors associated with shear-dependent platelet hyper-reactivity in patients on chronic aspirin therapy. *Thromb. Res.* **2010**, *126*, 379–383. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2975867/ (accessed on 17 May 2019). [CrossRef]
10. Hayward, C.P.; Harrison, P.; Cattaneo, M.; Ortel, T.L.; Rao, A.K. Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. *J. Thromb. Haemost.* **2006**, *4*, 312–319. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/j.1538-7836.2006.01771.x (accessed on 17 May 2019). [CrossRef]
11. Vázquez-Santiago, M.; Ziyatdinov, A.; Pujol-Moix, N.; Brunel, H.; Morera, A.; Soria, J.M.; Souto, J.C. Age and gender effects on 15 platelet phenotypes in a Spanish population. *Comput. Biol. Med.* **2016**, *69*, 226–233. Available online: https://www.sciencedirect.com/science/article/pii/S0010482515004163?via%3Dihub (accessed on 17 May 2019). [CrossRef] [PubMed]
12. Lippi, G.; Franchini, M. Laboratory screening for abnormalities of primary hemostasis: what's next? *Clin. Chem.* **2001**, *47*, 2071. Available online: http://clinchem.aaccjnls.org/content/47/11/2071.2.long (accessed on 17 May 2019). [PubMed]
13. Moeller, A.; Weippert-Kretschmer, M.; Prinz, H.; Kretschmer, V. Influence of *ABO* blood groups on primary hemostasis. *Transfusion* **2001**, *41*, 56–60. Available online: https://onlinelibrary.wiley.com/doi/abs/10.1046/j.1537-2995.2001.41010056.x?sid=nlm%3Apubmed (accessed on 17 May 2019). [CrossRef] [PubMed]
14. Favaloro, E.J.; Thom, J.; Patterson, D.; Just, S.; Baccala, M.; Dixon, T.; Meiring, M.; Koutts, J.; Rowell, J.; Baker, R. Potential supplementary utility of combined PFA-100 and functional von Willebrand factor testing for the laboratory assessment of desmopressin and factor concentrate therapy in von Willebrand disease. *Blood Coagul. Fibrinolysis* **2009**, *20*, 475–483. Available online: https://journals.lww.com/bloodcoagulation/Abstract/2009/09000/Potential_supplementary_utility_of_combined.17.aspx (accessed on 21 June 2019). [CrossRef] [PubMed]

15. Souto, J.C.; Almasy, L.; Borrell, M.; Blanco-Vaca, F.; Mateo, J.; Soria, J.M.; Coll, I.; Felices, R.; Stone, W.; Fontcuberta, J.; et al. Genetic susceptibility to thrombosis and its relationship to physiological risk factors: The GAIT study. Genetic Analysis of Idiopathic Thrombophilia. *Am. J. Hum. Genet.* **2000**, *67*, 1452–1459. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1287922/ (accessed on 17 May 2019). [CrossRef] [PubMed]
16. Pujol-Moix, N.; Vázquez-Santiago, M.; Morera, A.; Ziyatdinov, A.; Remacha, A.; Nomdedeu, J.F.; Fontcuberta, J.; Soria, J.M.; Souto, J.C. Genetic determinants of platelet large-cell ratio, immature platelet fraction, and other platelet-related phenotypes. *Thromb. Res.* **2015**, *136*, 361–366. Available online: https://www.thrombosisresearch.com/article/S0049-3848(15)30031-1/fulltext (accessed on 17 May 2019). [CrossRef]
17. Smith, N.L.; Chen, M.H.; Dehghan, A.; Strachan, D.P.; Basu, S.; Soranzo, N.; Hayward, C.; Rudan, I.; Sabater-Lleal, M.; Bis, J.C.; et al. Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation* **2010**, *121*, 1382–1392. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2861278/ (accessed on 17 May 2019). [CrossRef]
18. Sabater-Lleal, M.; Huffman, J.E.; de Vries, P.S.; Marten, J.; Mastrangelo, M.A.; Song, C.; Pankratz, N.; Ward-Caviness, C.K.; Yanek, L.R.; Trompet, S.; et al. Genome-Wide Association Transethnic Meta-Analyses Identifies Novel Associations Regulating Coagulation Factor VIII and von Willebrand Factor Plasma Levels. *Circulation* **2019**, *5*, 620–635. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6438386/ (accessed on 17 May 2019). [CrossRef]
19. Bunimov, N.; Fuller, N.; Hayward, C.P. Genetic loci associated with platelet traits and platelet disorders. *Semin. Thromb. Hemost.* **2013**, *39*, 291–305. Available online: https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-0033-1334466 (accessed on 17 May 2019). [CrossRef]
20. Chen, M.H.; Yanek, L.R.; Backman, J.D.; Eicher, J.D.; Huffman, J.E.; Ben-Shlomo, Y.; Beswick, A.D.; Yerges-Armstrong, L.M.; Shuldiner, A.R.; O'Connell, J.R.; et al. Exome-chip meta-analysis identifies association between variation in ANKRD26 and platelet aggregation. *Platelets* **2017**, *28*, 1–10. Available online: https://www.tandfonline.com/doi/abs/10.1080/09537104.2017.1384538?journalCode=iplt20 (accessed on 17 May 2019). [CrossRef]
21. Stierlin, F.B.; Molica, F.; Reny, J.L.; Kwak, B.R.; Fontana, P. Pannexin1 Single Nucleotide Polymorphism and platelet reactivity in a cohort of cardiovascular patients. *Cell Commun. Adhes.* **2017**, *23*, 11–15. Available online: https://www.tandfonline.com/doi/full/10.1080/15419061.2017.1282469 (accessed on 17 May 2019). [CrossRef] [PubMed]
22. Sokol, J.; Skerenova, M.; Ivankova, J.; Simurda, T.; Stasko, J. Association of genetic variability in selected genes in patients with deep vein thrombosis and platelet hyperaggregability. *Clin. Appl. Thromb. Hemost.* **2018**, *24*, 1027–1032. Available online: https://journals.sagepub.com/doi/full/10.1177/1076029618779136?url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&rfr_dat=cr_pub%3Dpubmed (accessed on 17 May 2019). [CrossRef] [PubMed]
23. Johnson, A.D.; Yanek, L.R.; Chen, M.H.; Faraday, N.; Larson, M.G.; Tofler, G.; Lin, S.J.; Kraja, A.T.; Province, M.A.; Yang, Q.; et al. Genome-wide metaanalyses identifies seven loci associatedwith platelet aggregation in response to agonists. *Nat. Genet.* **2010**, *42*, 608–613. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3057573/ (accessed on 17 May 2019). [CrossRef] [PubMed]
24. Qayyum, R.; Becker, L.C.; Becker, D.M.; Faraday, N.; Yanek, L.R.; Leal, S.M.; Shaw, C.; Mathias, R.; Suktitipat, B.; Bray, P.F. Genome-wide association study of platelet aggregation in African Americans. *BMC Genet.* **2015**, *6*, 58. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4448541/ (accessed on 17 May 2019). [CrossRef] [PubMed]
25. Heit, J.A.; Armasu, S.M.; Asmann, Y.W.; Cunningham, J.M.; Matsumoto, M.E.; Petterson, T.M.; De Andrade, M. A genome-wide association study of venous thromboembolism identifies risk variants in chromosomes 1q24.2 and 9q. *J. Thromb. Haemost.* **2012**, *10*, 1521–1531. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3419811/ (accessed on 17 May 2019). [CrossRef] [PubMed]

26. Tang, W.; Teichert, M.; Chasman, D.I.; Heit, J.A.; Morange, P.E.; Li, G.; Pankratz, N.; Leebeek, F.W.; Paré, G.; de Andrade, M.; et al. A genome-wide association study for venous thromboembolism: The extended cohorts for heart and aging research in genomic epidemiology (CHARGE) consortium. *Genet. Epidemiol.* **2013**, *37*, 512–521. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3990406/ (accessed on 17 May 2019). [CrossRef] [PubMed]
27. Germain, M.; Saut, N.; Greliche, N.; Dina, C.; Lambert, J.C.; Perret, C.; Cohen, W.; Oudot-Mellakh, T.; Antoni, G.; Alessi, M.C.; et al. Genetics of venous thrombosis: Insights from a new genome wide association study. *PLoS ONE* **2011**, *6*, e25581. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3181335/ (accessed on 17 May 2019). [CrossRef]
28. Trégouët, D.A.; Heath, S.; Saut, N.; Biron-Andreani, C.; Schved, J.F.; Pernod, G.; Galan, P.; Drouet, L.; Zelenika, D.; Juhan-Vague, I.; et al. Common susceptibility alleles are unlikely to contribute as strongly as the FV and *ABO* loci to VTE risk: Results from a GWAS approach. *Blood* **2009**, *113*, 5298–5303. Available online: http://www.bloodjournal.org/content/113/21/5298.long?sso-checked=true (accessed on 17 May 2019). [CrossRef]
29. Gorski, M.M.; de Haan, H.G.; Mancini, I.; Lotta, L.A.; Bucciarelli, P.; Passamonti, S.M.; Cairo, A.; Pappalardo, E.; van Hylckama Vlieg, A.; Martinelli, I.; et al. Next-generation DNA sequencing to identify novel genetic risk factors for cerebral vein thrombosis. *Thromb. Res.* **2018**, *169*, 76–81. Available online: https://www.thrombosisresearch.com/article/S0049-3848(18)30385-2/fulltext (accessed on 17 May 2019). [CrossRef]
30. Manco, L.; Silva, C.; Fidalgo, T.; Martinho, P.; Sarmento, A.B.; Ribeiro, M.L. Venous thromboembolism risk associated with ABO, F11 and FGG loci. *Blood Coagul. Fibrinolysis* **2018**, *6*, 528–532. Available online: https://insights.ovid.com/pubmed?pmid=29995659 (accessed on 17 May 2019). [CrossRef]
31. Reilly, M.P.; Li, M.; He, J.; Ferguson, J.F.; Stylianou, I.M.; Mehta, N.N.; Burnett, M.S.; Devaney, J.M.; Knouff, C.W.; Thompson, J.R.; et al. Identification of ADAMTS7 as a novel locus for coronary atherosclerosis and association of *ABO* with myocardial infarction in the presence of coronary atherosclerosis: Two genome-wide association studies. *Lancet* **2011**, *377*, 383–392. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3297116/ (accessed on 17 May 2019). [CrossRef]
32. Williams, F.M.; Carter, A.M.; Hysi, P.G.; Surdulescu, G.; Hodgkiss, D.; Soranzo, N.; Traylor, M.; Bevan, S.; Dichgans, M.; Rothwell, P.M.; et al. Ischemic stroke is associated with the *ABO* locus: The EuroCLOT study. *Ann. Neurol.* **2013**, *73*, 16–31. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3582024/ (accessed on 22 Jun 2019). [CrossRef] [PubMed]
33. Ling, X.; Zheng, Y.; Tao, J.; Zheng, Z.; Chen, L. Association study of polymorphisms in the *ABO* gene with ischemic stroke in the Chinese population. *BMC Neurol.* **2016**, *16*, 146. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4992279/ (accessed on 17 May 2019). [CrossRef] [PubMed]
34. IBC 50K CAD Consortium. Large-scale gene-centric analysis identifies novel variants for coronary artery disease. *PLoS Genet.* **2011**, *7*, e1002260. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3178591/ (accessed on 17 May 2019).
35. Schunkert, H.; König, I.R.; Kathiresan, S.; Reilly, M.P.; Assimes, T.L.; Holm, H.; Preuss, M.; Stewart, A.F.; Barbalic, M.; Gieger, C.; et al. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. *Nat. Genet.* **2011**, *43*, 333–338. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3119261/ (accessed on 17 May 2019). [CrossRef] [PubMed]
36. Bruzelius, M.; Strawbridge, R.J.; Trégouët, D.A.; Wiggins, K.L.; Gertow, K.; Sabater-Lleal, M.; Öhrvik, J.; Bergendal, A.; Silveira, A.; Sundström, A.; et al. Influence of coronary artery disease-associated genetic variants on risk of venous thromboembolism. *Thromb. Res.* **2014**, *134*, 426–432. Available online: https://www.thrombosisresearch.com/article/S0049-3848(14)00184-4/fulltext (accessed on 17 May 2019). [CrossRef] [PubMed]
37. Franchini, M.; Lippi, G. Relative risks of thrombosis and bleeding in different *ABO* blood groups. *Semin. Thromb. Hemost.* **2016**, *42*, 112–117. Available online: https://www.thieme-connect.com/products/ejournals/abstract/10.1055/s-0035-1564832 (accessed on 17 May 2019). [PubMed]
38. Zhang, H.; Mooney, C.J.; Reilly, M.P. ABO blood groups and cardiovascular diseases. Review Article. *Int. J. Vasc. Med.* **2012**, *2012*, 641917. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3485501/ (accessed on 17 May 2019). [PubMed]

39. Barbalic, M.; Dupuis, J.; Dehghan, A.; Bis, J.C.; Hoogeveen, R.C.; Schnabel, R.B.; Nambi, V.; Bretler, M.; Smith, N.L.; Peters, A.; et al. Large-scale genomic studies reveal central role of *ABO* in sP-selectin and sICAM-1 levels. *Hum. Mol. Genet.* **2010**, *19*, 1863–1872. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2850624/ (accessed on 17 May 2019). [CrossRef]
40. Souto, J.C.; Almasy, L.; Muñiz-Diaz, E.; Soria, J.M.; Borrell, M.; Bayén, L.; Mateo, J.; Madoz, P.; Stone, W.; Blangero, J.; et al. Functional effects of the *ABO* locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. *Arterioscler. Thromb. Vasc. Biol.* **2000**, *20*, 2024–2028. Available online: https://www.ahajournals.org/action/doSearch?AllField=Souto+JC&publication=atvb (accessed on 17 May 2019). [CrossRef]
41. Chen, Y.; Chen, C.; Ke, X.; Xiong, L.; Shi, Y.; Li, J.; Tan, X.; Ye, S. Analysis of circulating cholesterol levels as a mediator of an association between *ABO* blood group and coronary heart disease. *Circ. Cardiovasc. Genet.* **2014**, *7*, 43–48. Available online: https://www.ahajournals.org/doi/full/10.1161/CIRCGENETICS.113.000299?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3dpubmed (accessed on 17 May 2019). [CrossRef] [PubMed]
42. Paterson, A.D.; Lopes-Virella, M.F.; Waggott, D.; Boright, A.P.; Hosseini, S.M.; Carter, R.E.; Shen, E.; Mirea, L.; Bharaj, B.; Sun, L.; et al. Genome-wide association identifies the *ABO* blood group as a major locus associated with serum levels of soluble E-selectin. *Arterioscler. Thromb. Vasc. Biol.* **2009**, *29*, 1958–1967. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3147250/ (accessed on 17 May 2019). [CrossRef] [PubMed]
43. Zhong, M.; Zhang, H.; Reilly, J.P.; Chrisitie, J.D.; Ishihara, M.; Kumagai, T.; Azadi, P.; Reilly, M.P. ABO blood group as a model for platelet glycan modification in arterial thrombosis. *Arterioscler. Thromb. Vasc. Biol.* **2015**, *35*, 1570–1578. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4521621/ (accessed on 17 May 2019). [CrossRef] [PubMed]
44. Zhong, X.; Kriz, R.; Seehra, J.; Kumar, R. N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression. *FEBS Lett.* **2004**, *562*, 111–117. Available online: https://febs.onlinelibrary.wiley.com/doi/full/10.1016/S0014-5793%2804%2900191-7?sid=nlm%3Apubmed (accessed on 17 May 2019). [CrossRef]
45. Miller, S.A.; Dykes, D.D.; Polesky, H.F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **1988**, *16*, 1215. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC334765/ (accessed on 17 May 2019). [CrossRef] [PubMed]
46. Olsson, M.L.; Chester, M.A. A rapid and simple *ABO* genotype screening method using a novel B/O2 versus A/O2 discriminating nucleotide substitution at the *ABO* locus. *Vox Sang.* **1995**, *69*, 242–247. Available online: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1423-0410.1995.tb02602.x?sid=nlm%3Apubmed (accessed on 17 May 2019). [PubMed]
47. Delaneau, O.; Zagury, J.-F.; Marchini, J. Improved whole-chromosome phasing for disease and population genetic studies. *Nat. Methods* **2013**, *10*, 5–6. Available online: https://www.nature.com/articles/nmeth.2307 (accessed on 17 May 2019). [CrossRef]
48. Howie, B.N.; Donnelly, P.; Marchini, J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet.* **2009**, *5*, e1000529. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2689936/ (accessed on 17 May 2019). [CrossRef]
49. Lynch, M.; Walsh, B. *Genetics and Analysis of Quantitative Traits*; Sinauer Associates Inc.: Sunderland, MA, USA, 1998.
50. Shabalin, A.A. Matrix eQTL: Ultra fast eQTL analysis via large matrix operations. *Bioinformatics* **2012**, *28*, 1353–1358. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3348564/ (accessed on 17 May 2019). [CrossRef]
51. Ziyatdinov, A.; Brunel, H.; Martinez-Perez, A.; Buil, A.; Perera, A.; Soria, J.M. Solarius: An R interface to SOLAR for variance component analysis in pedigrees. *Bioinformatics* **2016**, *32*, 1901–1902. Available online: https://academic.oup.com/bioinformatics/article/32/12/1901/1744140 (accessed on 17 May 2019). [CrossRef]

52. Almasy, L.; Blangero, J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am. J. Hum. Genet.* **1998**, *62*, 1198–1211. Available online: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1377101/ (accessed on 17 May 2019). [CrossRef] [PubMed]
53. Self, S.G.; Liang, K.Y. Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. *J. Am. Stat. Assoc.* **1987**, *82*, 605–610. [CrossRef]

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CAPÍTOL 5

DISCUSSIÓ:

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DISCUSSIÓ:

Sota el punt de vista anatomopatològic, la trombosi arterial es caracteritza per la presència de grans quantitats de plaquetes (coàgul blanc). Aquestes, conjuntament amb altres factors, tenen un paper fonamental en el desenvolupament de la placa d'ateroma, característica que defineix l'aterosclerosi i les seves malalties derivades sobretot a nivell de la circulació cardíaca, cerebral i de vasos perifèrics. Com s'ha descrit en aquesta tesi, l'aterosclerosi és una manifestació clínica de la disfunció endotelial a llarg termini [5]. S'ha postulat en treballs en ratolins amb hipercolesterolèmia que l'ADAMTS13 modula la progressió de la placa d'ateroma en un mecanisme depenent del FvW [32]. En contrapartida, no hi ha evidències del paper de les plaquetes en la trombosi venosa. Recentment s'ha descrit, en estudis experimentals amb ratolins [7], la presència d'agregats plaquetaris en el coàgul venós. En aquest estudi [7] es fa palès que les plaquetes i el FvW són crítics per la formació i el desenvolupament del coàgul venós. Tot això ens va portar a estudiar el paper de les plaquetes en la trombosi venosa.

5.1 HIPERREACTIVITAT PLAQUETÀRIA I TROMBOSI VENOSA:

La reactivitat plaquetària (hipo i hiperreactivitat plaquetària) es pot mesurar a) mitjançant l'agregació plaquetària activada per agonistes en PRP o b) mitjançant test que usen sang total. Aquests test en sang total poden ser o bé agregacions en sang total (usem l'analitzador Multiplate®) o bé mitjançant test basats en l'adhesió plaquetària (usem l'analitzador PFA-100®). Hem mesurat la reactivitat plaquetària d'ambdues maneres, ens referirem per això en termes de hiper o hipoagregabilitat plaquetària quan ho mesurem mitjançant l'agregació plaquetària.

Hi ha força evidències que la reactivitat plaquetària augmentada pot identificar-se en individus amb risc de trombosi arterial com infart de miocardi, accident cerebrovascular, i malaltia arterial perifèrica [33]. Però la relació entre la hiperagregabilitat plaquetària i la trombosi venosa és controvertida. Hi ha estudis que no troben relació, com per exemple en l'estudi de Framingham

Heart [34] o en agregació plaquetària en sang total mitjançant l'analitzador Multiplate® [20], i en altres sí en troben [35-37]. Aquests últims estudis són realitzats en mostres petites, a més a més, o bé no tenen controls o en tenen molts pocs. Els descriuré en més detall: Hayes, *et al* [35] analitza la hiperagregabilitat plaquetària en 64 pacients amb trombosi venosa, 31 pacients amb trombosi arterial i 14 voluntaris sans. En l'estudi es combinen els resultats dels pacients amb trombosi venosa i arterial. Per altra banda s'avalua la hiperagregabilitat amb tres agonistes diferents (ADP, EPI i àcid araquidònic) troben associació només entre la hiperagregabilitat induïda per EPI i la trombosi. Weber, *et al* [36] analitza la hiperagregabilitat plaquetària en 34 pacients amb trombosi venosa vs 53 controls sans; avaluen 4 agonistes (trombina (TRAP-6), col·lagen, ADP i EPI), troben associació entre trombosi venosa i hiperagregabilitat quan és induïda per trombina, no quan ho és per ADP o EPI. Per últim en l'estudi de Tekgündüz, *et al* [37] s'analitza la hiperagregabilitat plaquetària en 28 pacients amb trombosi venosa però aquest estudi no inclou controls.

El nostre treball amb la població RETROVE inclou un nombre molt més elevat d'individus (400 pacients amb trombosi venosa i 400 controls sans). S'analitza la hiperagregabilitat plaquetària induïda per ADP i EPI. La principal conclusió que vam obtenir fou que no hi havia associació entre la hiperagregabilitat plaquetària i el risc de trombosi venosa. Tal i com s'ha descrit [18,19] la hiperagregabilitat plaquetària es dóna també en la població normal. Nosaltres vam calcular la concentració d'agonista, tant d' EPI com d'ADP, que separa les dues poblacions (individus amb hipoagregabilitat plaquetària i individus amb hiperagregabilitat plaquetària), vam obtenir una concentració (0.5μM) molt semblant a les descrites per Yee, *et al* [18] i Berger, *et al* [19]. Els nostres resultats suggereixen que la hipo o hiperagregabilitat plaquetària no són significativament diferents en controls sans que en pacients amb trombosi venosa. Vam trobar diferències estadísticament significatives entre pacients i controls quan mesuràvem la agregabilitat plaquetària mitjançant l'àrea sota la corba, però aquestes diferències es van perdre quan vam ajustar-la per edat. Així observem que la hiperagregabilitat plaquetària sembla incrementar-se amb l'edat de la mateixa manera en pacients que en controls. També trobem que la hiperagregabilitat plaquetària està relacionada amb nivells alts de fibrinogen i escurçament dels temps d'oclusió del PFA-100®, però aquest comportament és el mateix en controls i en pacients. Yee, *et al* troben també nivells alts de

fibrinogen [18] i temps curts d'oclusió del PFA-100® [22] en individus sans amb hiperagregabilitat plaquetària, però aquests treballs no inclouen pacients amb trombosi.

En canvi, en estudis recents, el nostre grup ha trobat associació entre la hiperreactivitat plaquetària mesurada mitjançant el PFA-100® (temps escurçats d'oclusió) i la trombosi venosa. Això ens va portar a seguir investigant sobre la hiperreactivitat plaquetària, així vam estudiar diferents fenotips implicats en la fisiologia de l'agregació plaquetària com les biomolècules VAMP8, SERT i Serotonina.

Vam trobar associació amb trombosi venosa en dones amb nivells alts de VAMP8 i SERT. Nivells elevats d'aquestes proteïnes s'havien associat a trombosi arterial [19,21] però no hi havia evidència de la seva implicació en la trombosi venosa. Així doncs, el nostre, és el primer treball que troba aquesta associació. Diferents treballs troben polimorfismes de VAMP8 associats a malaltia cardiovascular: per exemple el polimorfisme r*s1561198* [38] o el polimorfisme *rs 1010* [21,23]. També diversos estudis suggereixen una connexió entre els mecanismes serotonèrgics i els episodis cardiovasculars. Hi ha doncs evidència que un polimorfisme del promotor de *SLC6A4* (gen del SERT) està associat a infart de miocardi [24,25]. Nosaltres no vam trobar associació d'aquests dos últims polimorfismes amb la trombosi venosa. Sovint, polimorfismes associats a trombosi arterial no ho estan a trombosi venosa.

Vam estudiar els nivells de VAMP8, SERT i Serotonina després de classificar els individus segons l'agregabilitat plaquetària (individus amb hipo o hiperagregabilitat plaquetària). Vam trobar associació entre els nivells alts d'aquestes biomolècules i la trombosi venosa en les dones amb hiperagregabilitat plaquetària. Els nostres resultats estan d'acord amb resultats previs on els nivells alts de VAMP8 i SERT s'han associat a la hiperreactivitat plaquetària i aquesta s'ha associat en dones [18]. No trobem associació entre els nivells de serotonina i la trombosi venosa. En canvi quan analitzem la serotonina en els individus hiperreactius sí trobem associació amb la trombosi venosa. Això indicaria una clara relació dels nivells de serotonina i l'agregació plaquetària.

Quan el nostre grup va descriure que els temps curts d'oclusió del PFA-100® estan associats a trombosi venosa, [20] va suggerir que el mecanisme majoritari implicat en aquesta relació podria ser l'adhesió plaquetària. Els nostres estudis semblen corroborar aquesta hipòtesi. Així, trobem que l'agregació plaquetària explica només el 12% dels valors curts de PFA-100®. Hayes, *et al* [35] no va trobar correlació entre la hiperagregabilitat plaquetària i els valors de PFA-100®. Per altra banda no hem trobat associació entre els temps curts de PFA-100® i els nivells de SERT, VAMP8 i Serotonina (biomolècules associades a agregació plaquetària). Els nostres resultats recolzen doncs la hipòtesi que els PFAs curts podrien ser deguts a anomalies en l'adhesió plaquetària.

Hi ha alguns estudis que mesuren la hiperagregabilitat plaquetària mitjançant agregació en sang total. El nostre grup, com s'ha descrit anteriorment, no va trobar associació entre els paràmetres d' agregació en sang total i la trombosi venosa [20]. Per contra, Campello, *et al* [39] va detectar, en pacients obesos, hipercoagulabilitat mitjançant tromboelastometria en sang total (ROTEM) i agregometria per impedància (Multiplate®). L'analitzador Multiplate® és útil per monitoritzar la resposta de l'antiagregació per fàrmacs antiplaquetaris. Aquests fàrmacs són àmpliament usats en malaltia cardiovascular. Així diversos estudis mesuren, mitjançant l'analitzador Multiplate®, la resposta a fàrmacs antiplaquetaris en pacients amb trombosi arterial [40, 41]. Troben que els individus que no responen al clopidogrel tenen una resposta a la agregació plaquetària més alta que els individus que sí en responen quan han estat estimulats amb àcid araquidònic o trombina (TRAP) indicant doncs una hiperreactivitat plaquetar general.

Nosaltres hem trobat que l'analitzador Multiplate® no distingeix entre individus amb hipo o hiperagregabilitat plaquetar, possiblement per que la corba d'agregació és menys sensible o bé les concentracions no són les idònies: tant Hayward [42] com el nostre grup, no va trobar resposta a l'agregació plaquetària en sang total a l'agonista EPI. Així doncs només vam mesurar l'agregació plaquetària amb l'agonista ADP. La concentració requerida per a mesurar l'agregació amb l'agonista ADP en sang total segons Hayward [42] hauria de ser 5 vegades superior a la mesurada mitjançant LTA, així doncs podria ser que la concentració de 0,5μM no fos l'adequada. Per l'altra banda, l'agregometria per impedància no permet avaluar els canvis en la forma de la

plaqueta, Sokol, *et al* [43] suggereix només l'ús de LTA per a mesurar la hiperagregabilitat plaquetària.

Podem concloure que no trobem associació entre la hiperagregabilitat plaquetària i la trombosi venosa i que els nostres resultats suggereixen una possible associació dels mecanismes d'adhesió amb la trombosi venosa.

Recentment s'han obert altres vies d'estudi en l'associació de la hiperreactivitat plaquetària i la trombosi: la microbiota. Alguns nutrients que s'obtenen de la dieta com fosfatidilcolina, colina i carnitina són processats específicament per la microbiota intestinal produint trimetilamina (TMA). TMA és absorbit per l'intestí i convertit al fetge en trimetilamina N- òxid (TMAO). S'ha observat que la microbiota a través de la generació de TMAO directament contribueix a la hiperreactivitat plaquetària i augmenta el potencial trombòtic [44,45]. L'exposició directa de les plaquetes a nivells elevats de TMAO estan associats a un alliberament de Ca^{2+} intracel·lular. Aquest Ca^{2+} augmenta l'activació plaquetària depenent de l'estímul de múltiples agonistes.

Zhu, *et al*. [44] descriu que la microbiota, mitjançant el TMAO, participa en la modulació de la funció plaquetar i la generació d'un fenotip pro trombòtic *in vivo.* Suggereix una estratègia de tractament pels individus amb trombosi arterial basada en el tractament de la microbiota (manipulació dietètica, probiòtics o prebiòtics o directament inhibidors farmacològics d'enzims involucrats en la producció de TMA).

5.2 HEMOSTÀSIA PRIMÀRIA I DONES:

Les plaquetes expressen receptors per l'estrogen que poden afectar la funció de les plaquetes i la seva habilitat hemostàtica. S'ha observat una major funció plaquetària durant l'embaràs [46,47]. Per altra banda els estrògens potencien l'agregació plaquetària induïda per trombina [48]. Així doncs la funció plaquetària pot ser diferent en relació a l'edat, el gènere i el cicle menstrual.

Sembla que la hiperagregabilitat plaquetària pot ser necessària per les dones, ja que tenen mensualment la menstruació i a vegades tenen sagnats fatals durant el període perinatal. Haque, *et al* [49] suggereixen que així com els estrògens

potencien l'agregació plaquetària la testosterona en redueix l'agregació. Troben que les dones tenen una agregabilitat plaquetària més gran que els homes. Nosaltres vam trobar que la hiperagregabilitat plaquetària s'associava a risc trombòtic en les dones però malauradament es perdia quan ho ajustàvem per l'edat.

En els nostres estudis (capítol 4.2 (VAMP8 i SERT) i capítol 4.3 (ADAMTS13)) trobem una associació d'aquestes proteïnes amb la trombosi venosa principalment en les dones.

Així doncs trobem que en població espanyola, els nivells augmentats de VAMP8 i SERT s'associen, de manera independent, a un major risc trombòtic venós en les dones. Berlin, *et al.* [50] troben una major agregació plaquetària en dones en edat fèrtil que en post menopàusiques i en homes. Podria ser una mecanisme per prevenir el sagnat menstrual fort. Nosaltres hem trobat nivells més alts de VAMP8, SERT i serotonina en dones en edat fèrtil que en menopàusiques.

D'altra banda també hem trobat que els nivells baixos d'ADAMTS13 s'associen a trombosi venosa en les dones. L'associació de nivells baixos d'ADAMTS13 amb trombosi arterial estava àmpliament estudiada [26-30] però l'associació amb trombosi venosa era més controvertida. Mazzetto, *et al* [31] observa nivells elevats de FvW associats amb nivells elevats d'ADAMTS13 en pacients amb trombosi venosa. Aquest estudi analitza la relació entre la inflamació i la trombosi i troba marcadors inflamatoris elevats en els pacients. Suggereix que els pacients poden presentar nivells elevats dels marcadors inflamatoris durant un llarg període de temps després de l'episodi agut de trombosi venosa. Aquest nivells elevats d'ADAMTS13 podrien ser deguts a un mecanisme compensatori contra els nivells persistentment elevats de FvW. Malauradament aquest estudi no es va avaluar per gènere. Recentment, altres estudis han trobat també associació de nivells baixos d'ADAMTS13 amb la trombosi venosa [51] però aquest estudi tampoc s'ha avaluat per gènere.

Els resultats obtinguts en el nostre estudi no es van replicar en la població RETROVE (dades no publicades), doncs si bé vam trobar associació de nivells baixos d'ADAMTS13 amb trombosi venosa en les dones, al ajustar per edat les diferències significatives es van perdre. En el projecte RETROVE els nivells

baixos d'ADAMTS13 per ells mateixos no semblen estar relacionats amb el risc de trombosi venosa però quan combinem els nivells baixos d' ADAMTS13 amb els nivells alts de FvW sí trobem una diferència estadísticament significativa entre pacients i controls que es manté quan s'ajusta per edat. Així doncs observem que l'associació entre els nivells alts de FvW i el risc trombòtic (OR= 8.48) augmenta notablement quan afegim els nivells disminuïts d'ADAMTS13 a l'anàlisi (OR=13.79). Es va observar que els pacients amb nivells disminuïts d'ADAMTS13 eren més vells i tenien FvW més elevats de manera estadísticament significativa. També es va observar que els nivells d'ADAMTS13 disminuïen amb l'edat (R= - 0.291, p< 0.001) i els de FvW augmentaven (R= 0.480, p< 0.001)). Aquests resultats són similars als d'Anwar, *et al* [52] que troba una correlació de R= -0.257, p< 0.001 entre l'edat i els nivells d'ADAMTS13, i una R= 0.269, p<0.001 entre l'edat i els nivells de FvW.

Per altra banda, el nostre grup va descriure que el recompte plaquetari (per sobre de $312x10^9$/L) s'associava a risc de trombosi venosa en dones suggerint que els estrògens podien jugar un rol en el major risc de trombosi venosa observat en les dones [53]. Bord, *et al.* [54] troben que els nivells d'estrogen durant la fase fol·licular del cicle menstrual s'associen a un increment del recompte plaquetari, mentre que el recompte plaquetari en dones post menopàusiques és més baix que en dones amb teràpia substitutiva d'estrogen [54].

A més a més, estudis recents [55,56] mostren que l'ADN mitocondrial (que s'hereta per via materna) s'allibera de les plaquetes activades i actua com a agonista per a induir l'activació de les plaquetes i la trombosi. Qin, *et al.* [56] descriu, en pacients amb bypass cardiopulmonar, l'associació de l'activació plaquetària amb nivells elevats d' ADN mitocondrial. Ells postulen que aquest ADN mitocondrial elevat que prové de l'activació de les plaquetes, juga un rol crític en la resposta inflamatòria dels pacients post- bypass cardiopulmonar.

5.3 HEMOSTÀSIA PRIMÀRIA I EL GRUP SANGUINI ABO:

Diversos estudis han documentat la influència del grup sanguini ABO sobre els nivells plasmàtics de FvW (els individus de grup sanguini O tenen els nivells de FvW un 25 % més baix que els de grup sanguini no-O [13-15]). El nostre grup va descriure, mitjançant estudis de lligament, que l'efecte del grup ABO sobre

els nivells de FvW eren deguts a un efecte funcional directe del locus *ABO* i que aquest estava en desequilibri de lligament a altres locus de regulació del FvW no identificats [11]. Alguns estudis també han valorat la influència del grup sanguini ABO en els nivells d'ADAMTS13 [52, 57].

S'ha observat la influència del grup sanguini ABO sobre la proteòlisi del FvW per l'ADAMTS13 [58, 59]. Aquests treballs troben que els nivells de FvW podrien incrementar la susceptibilitat de l'ADAMTS13 a trencar-lo en els portadors del grup sanguini O comparat amb els portadors del grup sanguini no-O. Així Bowen [58] descriu que la glicosilació del FvW pot ser decisiva en la proteòlisi per l'ADAMTS13: el domini A2 del FvW (el lloc de proteòlisi del FvW per l'ADAMTS13) està flanquejat per 7 llocs de glicosilació en la seva estructura primària, tanmateix en la seva estructura terciària el FvW pot tenir altres formes de glicosilació que poden ser rellevants. La presència de sucres en el grup sanguini O, A o B en un o més dels carbohidrats que flanquegen el lloc de glicosilació poden influir en la proteòlisi per exemple en un efecte de càrrega. En individus amb el grup sanguini O, el domini A2 del FvW adopta una conformació que és més permissiva per la proteòlisi de l'ADAMTS13. En canvi, els antígens A i B protegeixen contra la proteòlisi de FvW. En individus del grup sanguini O, el FvW és trencat més ràpidament per l'ADAMTS13. La susceptibilitat del FvW per ser degradat per l'ADAMTS13 sembla decréixer en l'ordre O>B>A>AB. En individus amb el fenotip de Bombay [59] , els quals no tenen antigen H, els nivells de FvW són similars o més baixos que els obtinguts en els individus amb el grup sanguini O. D'aquesta manera els individus amb fenotip Bombay mostren una susceptibilitat a la degradació del FvW mitjançant l'ADAMTS13 major que els individus del grup sanguini O. McGrath, *et al* [60] troba que el FvW que prové de les plaquetes té una glicoforma diferent del FvW que prové del plasma, d'aquesta manera el FvW plaquetar és més resistent a la proteòlisi per l'ADAMTS13.

En el nostre estudi vam trobar que els nivells de FvW depenien del grup sanguini mentre que els d'ADAMTS13 no. Anwar*, et al.* [52] van comparar, en població àrab, els nivells d'ADAMTS13 en individus de grup sanguini O i no-O, tampoc van trobar diferències estadísticament significatives. Els mateixos resultats va obtenir Rios, *et al* [57] en individus amb hemodiàlisi. Ambdós estudis comparen individus del grup sanguini no-O i O, però cap d'ells distingeix entre els genotip *A1* i *A2* com sí ho vam discriminar en el nostre treball.

S'ha postulat que els nivells baixos de FvW en el grup sanguini O podrien ser deguts a nivells alts d'ADAMTS13, però nosaltres, d'acord amb Anwar, *et al*. [52] no trobem aquest resultat. Es suggereix que el que causa els nivells baixos de FvW no està relacionat amb canvis quantitatius en l'ADAMTS13, sinó que pot estar relacionat amb diferències estructurals a la proteïna de FvW.

Vam observar que el genotip A_2O es comportava com el genotip *O*, aquest resultat està d'acord amb resultats previs obtinguts pel nostre grup amb el genotip *ABO* i els nivells de FVIII [12]. O' Donell, *et al* [61] descriu que el nivell de l'activitat A-transferasa en els individus A_2O és baix. L'al·lel A_2 difereix de l'al·lel A_1 per la deleció d'un codó stop. Això fa que aquesta A-transferasa sigui una molècula més gran (per que té un domini carboxiterminal addicional) i sigui menys eficient en convertir les estructures bàsiques al grup sanguini A. Ells van observar que la quantitat d'antigen expressat per unitat de FvW era significativament més alta en A_1A_1 i A_2O que en individus *OO*. L'ordre dels nivells de FvW en funció del genotip *ABO* en la nostra població va ser: $O=A_2O<A1<B<AB$. Vam trobar que la distribució del FvW en funció del genotip *ABO* era independent del sexe i del grup d' individu (cas-control).

Per altra banda, el grup sanguini ABO també influencia els temps d'oclusió del PFA-100®. Així doncs, els individus del grup O tenen temps d'oclusió més llargs que el individus no-O [62-64]. Cosa que s'interpreta com una conseqüència que els individus del grup sanguini O tinguin nivells mes baixos de FvW [62-64]. Moeller, *et al* [65] troben una associació entre els temps d'oclusió del PFA-100® i els diferents grups sanguinis que decreixen en el següent ordre: O> A>B ≥AB. S'ha descrit que ambdós fenotips mesurats pel PFA-100® (PFA_EPI i PFA_ADP) tenen un fort component genètic, amb una heredabilitat de 0.45 per PFA_ADP i 0.52 per PFA_EPI [66]. El nostre treball descriu que el locus *ABO,* que està situat al cromosoma 9, és el principal determinant genètic del fenotip de PFA-100®. En el cas de PFA_EPI tota la influència d' *ABO* és mediada pel FvW i/o el FVIII. En el cas de PFA_ADP només part d'aquesta influència és mediada per FvW i/o FVIII.

La glicosilació sembla tenir un paper rellevant en la reactivitat plaquetar:

S’ha descrit que el grup sanguini ABO mitjançant la glicosilació pot influir en la reactivitat plaquetar [11, 67-72]. Així doncs els antígens A i B expressats en les glicoproteïnes plaquetaries poden modificar l’activitat dels complexes proteics d’adhesió plaquetària i de la interacció glicans-galactina. Les galactines són inductors plaquetars potents. Els glicoesfingolípids plaquetaris, que estan involucrats en l’agregació plaquetària i la trombosi mitjançant la unió de molècules d’adhesió cel·lulars, tenen també els antígens A i B.

La glicosilació però és també essencial per altres funcions plaquetars no relacionades amb el grup sanguini. Per exemple en la glicosilació del receptor $P2Y_{12}$. [73]. Aquest receptor conté dos llocs potencials de glicosilació al extrem amino-terminal. La pèrdua de glicosilació d’aquest receptor porta a una reactivitat plaquetar defectuosa, doncs es produeix una inhibició incompleta de l’activitat adenilil ciclasa mediada pel $P2Y_{12}$. El fet que el fenotip PFA_ADP no estigui totalment explicat pel FVIII i/o FvW pot suggerir que el locus *ABO* podria influir en la glicosilació del receptor $P2Y_{12}$.

Nagy, *et al* [74] descriu, mitjançant estudis amb ratolins, que el receptor $P2Y_{12}$ està substancialment involucrat en la hiperreactivitat plaquetària associada a hipercolesterolèmia.

Podem concloure que el grup sanguini ABO té una forta influència en l’hemostàsia primària i la trombosi, doncs determina els nivells de FvW i del fenotip PFA_EPI, ambdós fenotips associats a trombosi venosa.

CAPÍTOL 6

CONCLUSIONS:

CAPÍTOL 6

CONCLUSIONS:

1. La hiperagregabilitat plaquetària no està associada a trombosi venosa en la població RETROVE. La concentració d'inductor (tant per l'ADP com per l'EPI) que separa clarament els individus amb hipo i hiperagregabilitat plaquetària mesurada en PRP es de 0,5 μM.

2. L'agregació plaquetària induïda per EPI (EPI_AUC) i els valors del PFA-100® correlacionen amb una R=-0,342. Això indica que només el 12% del valors de PFA-100® són explicats pels valors d'agregació plaquetària.

3. En població espanyola, els nivells augmentats de VAMP8 i SERT s'associen, de manera independent, a un major risc de trombosi venosa en les dones. Mentre que els polimorfismes *rs 1010* de VAMP8 i *SLC6A4* de SERT no s'associen a risc de trombosi venosa.

4. En població espanyola, els nivells baixos d'ADAMTS13 s'associen, de manera independent, a un major risc de trombosi venosa en les dones. Els nivells de FvW depenen del grup sanguini mentre que els d'ADAMTS13 no. L'ordre dels nivells de FvW en funció del genotip *ABO* en la nostra població va ser: $O=A_2O<A1<B<AB$. La distribució del FvW en funció del genotip *ABO* és la mateixa tant en homes i dones com en controls o pacients.

5. El locus *ABO* es el principal determinant del fenotip de PFA-100®:

    1. En el cas de PFA_EPI tota la influència de *ABO* és mediada pel FvW i/o el FVIII.
    2. En el cas de PFA_ADP la influència del locus *ABO* és parcialment independent del FvW i/o FVIII, suggerint que altres mecanismes puguin explicar l'efecte *ABO* en aquest fenotip.

ANNEX:

Thrombosis Research 169 (2018) 57–63

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Short closure time values in PFA–100® are related to venous thrombotic risk. Results from the RETROVE Study

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ARTICLE INFO

Keywords:
PFA phenotypes
Closure times
Whole blood aggregation
Platelets
Venous thromboembolism

ABSTRACT

Introduction: Platelets play a role in the pathophysiology of venous thromboembolism (VTE). Some studies have not found an association between VTE and platelet aggregation. The PFA–100® analyser is an *in vitro* assay for assessing primary haemostasis. But, there are no studies to evaluate its association with VTE. We investigated the contribution of the global platelet function and aggregation in the development of VTE.

Material and methods: We analysed 800 individuals who were included in the RETROVE Study (Riesgo de Enfermedad TROmboembólica VEnosa). Global platelet function was evaluated as closure times (CT) with the agonists ADP and epinephrine using a PFA–100® analyser. Platelet aggregation was evaluated by Multiplate™ analyser. The VTE risk for all the parameters was calculated by unconditional logistic regression analyses considering the potential confounders: age, gender, body mass index (BMI), factor VIII (FVIII), the von Willebrand factor (vWF) and the ABO blood group system.

Results: The unadjusted *odds ratio* (OR) values ≤10th percentile for the PFAadp and PFAepi were 4.02 (95% CI, 2.76–5.95) and 3.33 (2.27–4.97). Also, after adjusting for vWF, we obtained lower OR for the PFAadp and for PFAepi: 2.24 (1.44–3.49) and 1.63 (1.04–2.59). But, the whole blood aggregation parameters did not shown an association with VTE risk.

Conclusion: We demonstrated an association between short CT and VTE risk. Although, the whole blood aggregation parameters did not show an association with the VTE risk. This striking contrast suggests that there are other platelet function mechanisms (*e.g.* adhesion) that are responsible of VTE risk.

1. Introduction

Venous thromboembolism (VTE) consists in Deep Vein Thrombosis (DVT) and Pulmonary Embolism (PE) and constitutes a leading cause of morbidity and mortality [1, 2]. Platelets, together with endothelial cells and circulating coagulation proteins, are crucial mediators of vascular haemostasis and, therefore, they play an important role in VTE [3].

The function of platelets in the pathophysiology of thrombosis is well established [4]. Injury in the endothelium leads to exposure of the circulating blood cells to collagen from the subendothelial space. Platelets, through their glycoproteins (GP) GPVI and GPIb–V–IX, interact with collagen and the von Willebrand factor (vWF) [5]. Collagen exposure leads to platelet adhesion and the formation of a platelet monolayer. Activated platelets recruit other circulating platelets, coagulation factors and other cell types by secreting aggregatory mediators.

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https://doi.org/10.1016/j.thromres.2018.07.012
Received 20 April 2018; Received in revised form 27 June 2018; Accepted 5 July 2018
Available online 11 July 2018
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Classically, platelet function has been considered very relevant in arterial thrombosis. However, recent studies have demonstrated their fundamental role in the mechanisms of VTE, at least in animal models [3, 6–8]. In addition, a review has described platelet features involved in the pathophysiology of VTE in humans [9].

An initial approach to assess platelet function begins with an appropriate clinical evaluation, platelet counts and the observation of peripheral blood cell morphology. Further laboratory testing includes platelet function assays and vWF analyses [10].

Generally, different methodologies are employed to evaluate platelet function such as bleeding time (BT), light transmission aggregometry on platelet rich plasma (PRP) or impedance aggregometry on whole blood [11]. Later, the PFA–100® analyser (Siemens Healthcare Diagnostics, Marburg, Germany) become a useful *in vitro* assay for the evaluation of overall platelet function [12–14].

The PFA–100® simulates a vessel wall under physiologic shear stress conditions. The device contains a membrane coated with collagen and also with ADP (PFAadp) or with epinephrine (PFAepi). This membrane has a hole through which the anticoagulated blood passes. The presence of these agonists and the high share rates results in platelet adhesion, activation and aggregation, and finally forming a stable platelet plug which occludes the hole. The time required to occlude the hole, in seconds, is reported as closure time (CT) and it is inversely proportional to the functional capacity of platelets [12].

The Multiplate™ analyser (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) is a multiple electrode impedance aggregometer that assesses platelet aggregation in whole blood [15, 16]. This method is based on the attachment of platelets to two platinum electrodes, resulting in an increase of electrical resistance between the electrodes. The change of resistance or impedance is continuously recorded and it is proportional to the amount of platelets stuck in the electrodes.

Several studies have attempted to develop models for predicting thrombogenicity and thrombus formation. The complex mechanisms behind high shear rate thrombus occlusion have been investigated by Mehrabadi et al. [17]. By using platelet function analyser, the PFA–100®, they developed a model for thrombus formation at high shear rates to predict thrombus growth and occlusion times.

Recently, the Framingham Heart Study [18], including 2831 individuals with venous thrombosis, has published results about baseline aggregability and VTE risk. An association between VTE risk and platelet aggregability was not found. In addition, a modest study [19], including 14 controls and 94 patients with venous thrombosis, has suggested a possible association between VTE risk and hyperaggregability at low concentration of the agonist (ADP and epinephrine). Despite these analyses, the risk of venous VTE has not been associated clearly with an elevated global platelet function and/or aggregation [18].

Considering the hypothesis of a relationship between platelet hyperreactivity and VTE, the aim of the present study is to investigate the role of the platelet function analysis, by means of PFA–100® and Multiplate™ parameters, in the assessment of VTE risk.

2. Material and methods

2.1. Study sample

Individuals were recruited from the RETROVE Study (Riesgo de Enfermedad TROmboembólica VEnosa) at the Hospital de la Santa Creu i Sant Pau, Barcelona (Spain) between 2012 and 2016. RETROVE is a prospective case–control study that included 400 consecutive adult (≥18 years) patients with VTE (according to specific clinical inclusion criteria) and 400 healthy volunteers who serve as controls. Detailed population baseline characteristics are described in Table 1. The goals of the RETROVE Study were to identify biomarkers for VTE and to establish mathematical algorithms to predict the risk of VTE. For this last objective, the control cohort was intentionally no matched neither in sex nor in age. In consequence, any statistical inference will always need an adjustment for these covariates.

The diagnosis of thrombosis was based on Doppler ultrasonography, tomography, magnetic resonance, arteriography, phlebography and pulmonary gammagraphy. Any type of thrombosis was included except those related to cancer. As detailed in Table 2, a VTE event was classified as either unprovoked or spontaneous or provoked or non–-spontaneous (one or more provoking factors within three months previous to an event) [20, 21]. In this last group, provoking factors were: surgery, immobilization, pregnancy or puerperium, oral contraceptives, prothrombotic non–neoplastic diseases and other circumstances. For the control group, 400 unpaid healthy volunteers (not blood bank donors and non–related with cases and neither among them) were included. They were recruited to match the age and sex distribution of Spanish population (2001 census).

Our study was performed in accordance with the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from all of the participants and all of the procedures were approved by the Institutional Review Board at the Hospital de la Santa Creu i Sant Pau.

2.2. Blood collection

Blood samples were taken at least 6 months after thrombosis to minimize the influence of the acute phase. None of the participants was taken oral anticoagulants within the previous 21 days, heparin in the previous 36 h or antiplatelet therapy or non–steroidal anti–inflammatory drugs (NSAIDs) in the previous week at the time of blood collection. Blood samples were collected from the antecubital vein between 9:00 a.m. and 12:00 p.m., under basal conditions and after 12–hour overnight fasting.

For the PFA analysis, all of the samples were drawn into 4.5 ml tubes that contained a 3.8% solution of sodium citrate as anticoagulant (BD Vacutainer Becton, Dickinson and Company, New Jersey, USA). To determine platelet aggregometry all of the samples were collected in tubes containing hirudin 25 μg/ml (Roche Diagnostics GmbH, Mannheim, Germany). Without exception, both testing were performed between 15 and 60 min after sampling.

2.3. Measurement of parameters

For the PFA analysis, the whole blood sample was transferred into the reservoir of the disposable test cartridges (PFAadp and PFAepi), already inserted in the instrument, and both CT were recorded. For Multiplate™ analysis, whole blood was added to the test cuvettes, stirred and warmed to 37 °C. The following aggregation values were recorded: area under the curve (AUC), maximum height of the aggregation (AGR) and maximum slope of the curve (SLP). Two different concentrations of the agonist ADP were used (ADP–test): 0.25 μM and 0.5 μM. We have considered only performing ADP–test because ASPI–test is used to support the monitoring and control of patients under antiplatelet therapy and not to study platelet aggregation through this receptor.

In our laboratory, the normal reference values for PFA–100® and Multiplate™ analysers were established in a previous sample of 80 healthy individuals. For the PFA analysis, the normal reference ranges CT values were comprised from 58 to 123 s for the PFAadp, and 72–191 s for the PFAepi. For the Multiplate™ analyser, the normal aggregability status was defined as the maximum aggregability (AGR 0.5 μM) values over 80%.

To measure plasma parameters, blood samples were collected with 1/10 volume of sodium citrate 0.13 M (BD Vacutainer Becton, Dickinson). Factor VIII (FVIII) levels were measured immediately after blood extraction. The Plasma–poor platelets (PPP) were obtained by centrifugation at 2000*g* for 20 min and stored at −20 °C until analysed. The FVIII activity was measured by STA®–Deficient FVIII (Stago,

Table 1
Clinical characteristics of patients and controls in the RETROVE Study.

Baseline characteristics[a]	Patients		Controls		$p^{\dagger}$
	Males	Females	Males	Females	
Gender (n)	196	204	194	206	
Age (year) (mean ± SD)	61.5 ± 16.9	66.2 ± 20.0	47.6 ± 17.7	50.5 ± 18.7	$< 2.2 \cdot 10^{-16}$
BMI (kg·m^{-2}) (mean ± SD)	28.0 ± 4.1	28.3 ± 6.1	26.0 ± 25.4	24.4 ± 4.1	$< 2.2 \cdot 10^{-16}$
Smoking (n, %)	37 (18.9)	18 (8.8)	40 (20.6)	35 (17.0)	NS
Alcohol consumption (n, %)	119 (60.7)	76 (34.3)	130 (67.0)	98 (47.6)	0.023
Hypertension (n, %)	83 (42.3)	94 (46.1)	35 (18.0)	40 (19.4)	$8.27 \cdot 10^{-15}$
Dyslipidaemia (n, %)	62 (31.6)	75 (36.8)	36 (18.6)	43 (20.9)	$3.46 \cdot 10^{-6}$
Statins (n, %)	46 (23.5)	60 (29.4)	18 (9.3)	32 (15.5)	$5.81 \cdot 10^{-7}$
Diabetes mellitus (n, %)	23 (11.7)	17 (8.3)	10 (5.2)	10 (4.9)	0.0073
Autoimmune disease (n, %)	20 (10.2)	21 (10.3)	14 (7.2)	18 (8.7)	NS
Ictus haemorrhagic (n, %)	0 (0.0)	2 (1.0)	1 (5.0)	0 (0.0)	NS
Arterial thrombosis background (n, %)	14 (7.1)	15 (7.4)	1 (5.0)	1 (5.0)	$1.08 \cdot 10^{-6}$
Non–steroidal anti–inflammatory drugs (n, %)	29 (14.8)	58 (28.4)	23 (11.9)	34 (16.5)	0.0058
Anti–platelet drugs (n, %)	24 (12.2)	18 (8.8)	2 (1.0)	5 (2.4)	$2.46 \cdot 10^{-7}$

[a] Number of individuals in each group. The percentages are given in parentheses. Individuals excluded by technical reasons are: 2 individuals for the BMI, 10 individuals for the ictus haemorrhagic and 10 individuals for the arterial thrombosis background.
[†] The statistical differences, fixed in a $p < 0.05$, are reported only for descriptive purposes. The non-significance is listed as NS.

Table 2
Characteristics of 400 consecutives thrombotic events in the RETROVE Study.

	Spontaneous			Non–spontaneous		
	M[a]	F[a]	Total[b]	M[a]	F[a]	Total[b]
Isolated deep vein thrombosis	82 (56.2)	63 (49.6)	145 (53.1)	27 (54.0)	38 (49.4)	65 (51.2)
No isolated deep vein thrombosis	30 (20.6)	19 (15.0)	49 (18.0)	10 (20.0)	11 (14.3)	21 (16.5)
Isolated pulmonary embolism	31 (21.2)	42 (33.1)	73 (26.7)	12 (24.0)	26 (33.8)	38 (29.9)
Visceral thrombosis	2 (1.4)	–	2 (0.7)	1 (2.0)	–	1 (0.8)
Venous sinus thrombosis	1 (0.7)	3 (2.4)	4 (1.5)	–	2 (2.6)	2 (1.6)
Total[c]	146 (36.5)	127 (31.8)	273 (68.3)	50 (12.5)	77 (19.3)	127 (31.8)

[a] The percentages between parentheses were obtained from the number of thrombosis types divided by the total number of males (M) or females (F).
[b] The percentages between parentheses were obtained from the number of thrombosis types divided by the total number of spontaneous or non–spontaneous events.
[c] The percentages between parentheses s were obtained from the number of male (M), female (F) or total divided by the total number of patients or controls.

Asnières sur Seine, France) coagulometric method. The vWF levels were measured by ELISA.

For the genetic analysis, DNA was isolated from peripheral blood leukocytes by a standard protocol [22]. Blood group genotyping was performed by PCR with subsequent enzyme digestion, as previously described [23].

2.4. Descriptive statistical analysis

The Chi–square test was used to compare pairs of categorical variables only for descriptive purposes. The non–parametric Mann–Whitney test was used to test differences between the mean values of the 2 platelet function, 6 Multiplate™ parameters and FVIII and vWF levels.

An unconditional logistic regression analysis was used to evaluate the VTE risk associated to PFA and Multiplate™ parameters. For the PFAadp and PFAepi, values ≤10th percentile of the controls were considered as risk factors. Also, we codified the individuals according to a new parameter called *redundant platelet function assay* (RPFA) parameter, which identifies individuals at risk when they have both PFAadp and PFAepi ≤10th percentile. For the Multiplate™ parameters, values ≥90th percentile of the controls were considered as risk factors. According to previous studies [24, 25] there are potential confounders that can introduce bias in determining risk factors for thrombosis. We used the Bayesian Information Criterion (BIC) to identify them [26]. For venous thrombosis, we found that age, sex, body mass index (BMI) and risk levels of FVIII and vWF (≥216% and ≥183%) were potential confounders. As published previously [27, 28], the *ABO locus* has functional effects on vWF and FVIII, therefore we considered the non–O blood group as a potential confounder.

Due to the potential effects of the potential confounders, we analysed the VTE risk using four statistical models for each parameters. The VTE risk models were: 1) unadjusted or crude (Model 1); 2) adjusted by age and sex (Model 2); and 3) adjusted by age, sex and vWF (Model 3). Finally, Model 4 was adjusted for age, sex, FVIII, vWF and ABO blood type, in the PFA parameters, and age, sex and BMI, in the Multiplate™ parameters. All of the variables included in the models are detailed in the Supplementary Table 1.

3. Results

3.1. Clinical characteristics in the RETROVE Study

We found statistically significant differences between patients and controls for age (61.7 ± 18.6 *vs* 47.1 ± 18.2 respectively; $p < 2.2 \cdot 10^{-16}$). Also, we reported differences for several categorical variables as shown in Table 1. According to our analysis of the 400 consecutive events of thrombosis, we observed more spontaneous (n = 273, 68.3%) than non–spontaneous VTE (n = 127, 31.8%) in our sample (Table 2).

The descriptive statistics of the PFA and Multiplate™ parameters are summarized in Table 3. We found significant differences between patients and controls only in the PFA parameters. For the PFAadp, the patients showed shorter CT values than the controls (77 ± 20.5 s *vs* 86 s ± 19.3 s; $p = 3.38 \cdot 10^{-16}$). Also, for the PFAepi, the patients showed shorter CT values than the controls (110 ± 30 s *vs*

Table 3
Descriptive statistics for the PFA, Multiplate™ and other parameters in the RETROVE Study.

	Patients[a]		Controls[a]		$p^{\dagger}$
	N	Mean ± SD	N	Mean ± SD	
PFA parameters[b]					
PFAadp (s)	397 (3)	77 ± 20.5	397 (3)	86 ± 19.3	$3.38\cdot10^{-16}$
PFAepi (s)	397 (3)	110 ± 30	397 (3)	127 ± 33.2	$1.15\cdot10^{-17}$
Multiplate™ parameters[b]					
AUC 0.25 μM (AU)	383 (17)	15.9 ± 9.5	383 (17)	15.2 ± 10.2	NS
AUC 0.5 μM (AU)	397 (3)	25.9 ± 14.7	389 (11)	24.8 ± 14.1	NS
AGR 0.25 μM (%)	385 (15)	31.4 ± 17.1	385 (15)	30.3 ± 18.2	NS
AGR 0.5 μM (%)	397 (3)	47.3 ± 25	390 (10)	45.2 ± 24.4	NS
SLP 0.25 μM (AU·min^{-1})	384 (16)	5.1 ± 1.8	385 (15)	4.93 ± 1.9	NS
SLP 0.5 μM (AU·min^{-1})	397 (3)	6.9 ± 3.0	390 (10)	6.7 ± 2.9	NS
Other parameters[b]					
PLA ($\times10^9$/L)[c]	400	236 ± 64	396 (4)	237 ± 55	NS
HCT (L/L)[c]	400	0.43 ± 0.04	396 (4)	0.42 ± 0.04	NS
HB (g/L)[c]	400	142 ± 15	396 (4)	142 ± 14	NS
FcVIIIC (%)	398 (2)	231.0 ± 84.1	397 (3)	143.2 ± 53.1	$2.32\cdot10^{-59}$
vWF (%)	399 (1)	200.7 ± 92.8	400	117.0 ± 53.2	$3.54\cdot10^{-52}$

[a] Numbers (N), means and standard deviations (SD) of the parameters. The PFA parameters are given in seconds (s). The Multiplate™ parameters are given in absorbance units (AU), percentages of aggregation (%) and absorbance units per minute (AU·min^{-1}).
[b] Number of the individuals analysed. The individuals excluded for technical reason are shown in parentheses.
[c] Platelet counts (PLA), haematocrit (HCT) and haemoglobin (HB).
[†] The statistical significances for the Mann–Whitney test are fixed as a $p < 0.05$. The non-significance is shown as NS.

147 ± 33.2 s; $p = 1.15\cdot10^{-17}$).

According to our normal reference values as mentioned in the Measurement of the parameters section, the RETROVE Study contains 20 healthy controls and 12 patients within CT PFAadp > 123 s and 18 healthy controls and 9 patients within CT PFAepi > 191 s. These individuals did not affect the VTE risk evaluation associated with the shortest CT values, on the opposite queue of the distribution.

To assess whether there was an association between CT and VTE risk, we stratified the sample into 4 groups (10th, 20th, 30th and 50th percentiles of the control distribution), as shown in Supplementary Table 2. Significant differences ≤20th percentile (72 s) for PFAadp and ≤50th percentile (120 s) for PFAepi were found. The shortest CT was

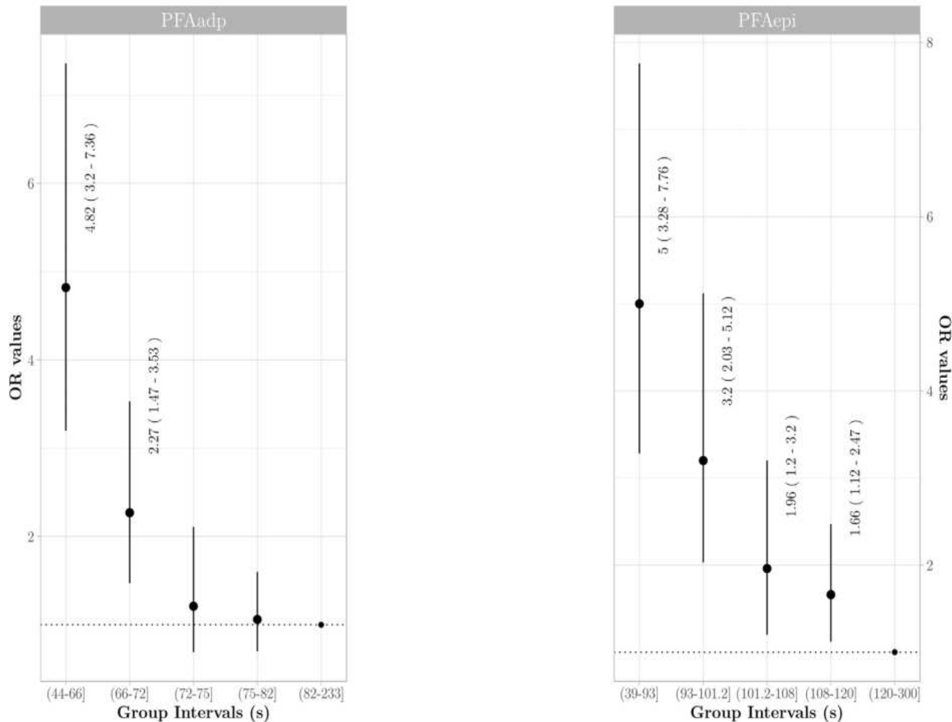

Fig. 1. Risk of venous thromboembolism (VTE) following Model 1 for the PFA adp and PFAepi. The *odds ratio* (OR) and the 95% confidence intervals (CI) with significant *p*–values are shown in the plot for the PFAadp (left) and the PFAepi (right). The rest of the OR and the 95% CI are shown in the Supplementary Table 2. The statistical significance of lower CI is fixed in 1.0, as indicated by the vertical broken line.

Table 4
Odds ratio (OR) of venous thromboembolism (VTE) events for the PFA parameters, under the 10th percentiles of controls, and Multiplate™ patameters, over the 90th percentiles of controls, in the RETROVE Study.

	Risk interval	Patients[a]	Controls[a]	Model 1[b]		Model 2[b]		Model 3[b]		Model 4[b]	
		N (%)	N (%)	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
PFAs											
PFAadp	(44–66)	128 (32.2)	42 (10.6)	4.02 (2.76–5.95)	$1.04 \cdot 10^{-12}$	3.36 (2.48–5.50)	$1.40 \cdot 10^{-10}$	2.24 (1.44–3.49)	$3.40 \cdot 10^{-4}$	2.90 (1.91–4.46)	$8.84 \cdot 10^{-7}$
PFAepi	(58–93]	110 (27.7)	41 (10.3)	3.33 (2.27–4.97)	$1.65 \cdot 10^{-9}$	2.87 (1.93–4.34)	$3.50 \cdot 10^{-7}$	1.63 (1.04–2.59)	0.035	1.97 (1.28–3.07)	0.002
RPFA	–	67 (16.9)	15 (3.8)	5.17 (2.98–9.56)	$2.64 \cdot 10^{-8}$	4.41 (2.49–7.58)	$1.06 \cdot 10^{-6}$	2.60 (1.38–5.13)	0.004	2.82 (1.52–5.48)	0.002
Multiplate™											
AUC 0.25 µM	[27.8–66)	37 (9.7)	39 (10.2)	0.94 (0.59–1.52)	NS	0.92 (0.55–1.51)	NS	0.93 (0.53–1.59)	NS	0.96 (0.57–1.59)	NS
AUC 0.5 µM	[47–79)	42 (10.6)	40 (10.3)	1.03 (0.65–0.89)	NS	0.97 (0.59–1.59)	NS	1.03 (0.6–1.74)	NS	0.93 (0.56–1.54)	NS
AGR 0.25 µM	[52.1–119)	39 (10.1)	39 (10.1)	1 (0.63–1.6)	NS	0.98 (0.59–1.61)	NS	1 (0.58–1.71)	NS	0.99 (0.59–1.64)	NS
AGR 0.5 µM	[81.8–150)	48 (12.1)	40 (10.3)	1.2 (0.77–1.88)	NS	1.12 (0.7–1.81)	NS	1.04 (0.61–1.75)	NS	1.09 (0.67–1.78)	NS
SLP 0.25 µM	[7.16–16.3)	43 (11.2)	39 (10.1)	1.12 (0.71–1.77)	NS	1 (0.61–1.63)	NS	0.94 (0.55–1.61)	NS	1.08 (0.66–1.78)	NS
SLP 0.5 µM	[11.1–21.8)	41 (10.3)	39 (10)	1.04 (0.65–1.65)	NS	0.98 (0.6–1.6)	NS	1.05 (0.61–1.79)	NS	0.93 (0.56–1.54)	NS

[a] Number of individuals (N) with the percentages (%) in each percentile. The number of individuals excluded for technical reasons are shown in parentheses in Table 3.
[b] The *odds ratios* (OR) within the 95% confidence intervals (CI) in parentheses for all the models. Model 1: non–adjusted model. Model 2: adjusted for age and sex. Model 3: adjusted for age, sex and von Willebrand factor (vWF). Model 4: adjusted for age, gender, factor VIII (FVIII), vWF and ABO blood group system, in the PFA parameters, and age, sex and body mass index (BMI), in the Multiplate™ parameters. The statistical significances are fixed as $p < 0.05$. The non-significance is shown as NS.

associated with an increased thrombosis risk, as shown in Fig. 1.

3.2. PFA parameters and VTE risk under 10th percentile

From the unconditional logistic regression analyses, we found a significant *odds ratio* (OR) for PFAadp, PFAepi and RPFA for the four statistical models as shown in Table 4. For these parameters, the highest ORs were obtained in the unadjusted model (Model 1). As expected in Model 1, the RPFA (OR = 5.17, 95% CI 2.98–9.56) showed the highest OR, because it represents the co–joint risk. In addition, the PFAadp (OR = 4.02, 95%, CI 2.76–5.95) and PFAepi (OR = 3.33, 95% CI 2.27–4.97) showed a significant OR. In Model 2, the OR values remained highly significant even after the adjustment for age and sex. In Model 3, when we added vWF, the OR values decreased, but they were still significant for all of the PFA parameters (OR = 2.24, 95% CI 1.44–3.49 for PFAadp; OR = 1.63, 95% CI 1.04–2.59 for PFAepi and OR = 2.60, 95% CI 1.38–5.13 for RPFA). Finally, all the parameters in the Model 4 (Fig. 2) showed a similar VTE risk as in Model 3 (Table 4).

3.3. Multiplate™ parameters and VTE risk over 90th percentile

As shown in Table 4 and Fig. 2, the Multiplate™ parameters (AUC 0.25 µM, AUC 0.5 µM, AGR 0.25 µM, AGR 0.5 µM, SLP 0.25 µM and SLP 0.5 µM) did not show any significant OR for any of the models.

4. Discussion

Our results show a clear association between short CT and VTE risk, for the usual agonists (ADP and epinephrine). Both agonists showed a strong association with VTE as the CT shortened (Fig. 1). It is interesting that we did not find an association between VTE risk and whole blood platelet aggregation.

To our knowledge, these analyses offer the first epidemiological evaluation between the risk of VTE and global platelet function, measured by the PFA–100® analyser. Classically, platelets are considered to play a pivotal role only in arterial thrombosis. Also, since arterial and venous thrombosis are related and share common risk factors [24, 25], our results highly suggest that an increased platelet function could be another shared risk factor.

According to our results, we did find a significant association between values of PFAadp ≤66 s and PFAepi ≤93 s and an increased VTE risk. When controlling for potential confounding, we observed a slight decrease in this VTE risk after adjusting for age and sex. In the RETROVE Study, it is important to emphasize that healthy controls were not matched with the patients for age and sex. Our cases are older than healthy controls. Despite this unavoidable difference, due to the design of the project, the findings on short PFAs are reliable, since in addition to the statistical control, we have previously published that PFAs are not influenced by age or sex in the Spanish general population [29]. To minimize the influence of additional covariates, we again adjusted our unconditional logistic regression models. Interestingly, we detected an abrupt decrease in the OR values after adjusting for vWF risk levels, although the associations with the VTE risk were still significant, for both parameters (Table 4). It is well–known that the vWF is involved in platelet adhesion [30, 31]. In addition, we observed a similar OR after adjusting for all of the potential confounders (Fig. 2). It's to say, incorporating ABO blood group and FVIII risk levels did not modify the results. Notably, the RPFA parameter that represent the co–joint risk from PFAadp and PFAepi, showed the strongest association with VTE risk. This suggests that the RPFA parameter is also a good marker for the risk of a thrombotic event, which has to be evaluated prospectively.

The PFA–100® analyser is used widely to assess the global platelet function, especially for the diagnosis of bleeding disorders [11, 13, 14, 32]. In addition, some studies have found elevated platelet hyperreactivity in arterial diseases [13, 33]. However, our results, strongly suggest that this methodology may be useful also in the field of venous thrombosis, just as it is for the diagnosis of bleeding disorders.

In our study, the platelet whole blood aggregation parameters measured by Multiplate™ analyser did not show an association with the

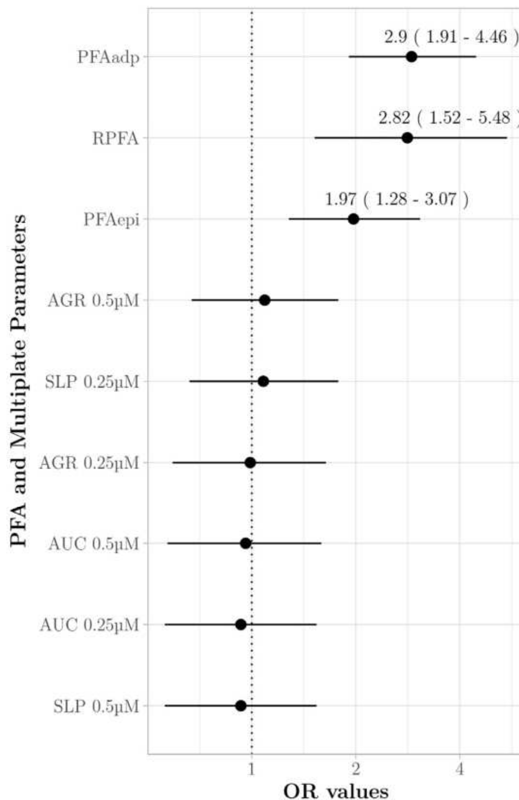

Fig. 2. Risk of venous thromboembolism (VTE) following the Model 4 for the PFA and Multiplate™ parameters. The *odds ratio* (OR) and the 95% confidence intervals (CI) with significant *p*–values are shown in the plot. The rest of the OR and the 95% CI are shown in the Table 4. The statistical significance of lower CI is fixed in 1.0, as indicated by the vertical broken line.

risk of VTE (Table 4, Fig. 2). While the PFA is a test performed under high shear forces where adhesions is a relevant factor influencing CT, the Multiplate™ platelet aggregometry is performed in a low shear environment. In agreement with our results, Puurunen et al. analyses [18] have not detected association between the VTE risk and platelet–rich plasma aggregation, measured by light transmission aggregometry. However, three small studies [19, 34, 35], have suggested a very weak association between the VTE risk and hyperaggregability in response to low–doses of agonists. According to previous studies [18] and our data, it seems that platelet aggregation is not related with the risk of VTE, at least in a significant way.

In support of our finding of the clinical relevance of platelet function on the risk of venous thromboembolism, recent clinical studies have demonstrated the efficacy of antiplatelet therapy (*e.g.* aspirin) to prevent the recurrence of VTE [36, 37]. A clinical trial comparing rivaroxaban with aspirin [38] also demonstrates the effect of antiplatelet therapy on the risk of venous thromboembolism. Despite rivaroxaban was more effective than aspirin for the prevention of recurrent VTE, the incidence of VTE in the aspirin treated group (4.4%) was lower than the incidence of recurrent VTE previously described (8.1%) [20].

All of these evidences indicate that there is an important relationship between the global platelet function and VTE risk. However, this relationship does not seem to be related to aggregation mechanisms. Our analyses, including vWF adjustment, suggest that the adhesion process could be responsible in part for the relationship, and for its role in the pathophysiology of VTE.

Finally, it is important to emphasize that our findings are subjected to some methodological limitations. Specifically, all the platelet parameters were measured at least 6 months after the thrombotic event. Consequently, we do not know if our results would be different if we studied patients during the acute phase. Another limitation comes from the case–control study design. The association found between PFA values and VTE risk could not be causal, since the measurement of platelet global function in the patients, necessarily was performed after the thrombotic event. The RETROVE study lacks data in some risk factors related to cardiovascular diseases, such as life style or regular sport activity.

5. Conclusion

In our analysis, we investigated the role of PFA and Multiplate™ parameters as risk factors for VTE. Our results reported that the VTE risk increases with shortest CT for PFAadp and PFAepi. Especially, shorter CT values for the PFA parameters (even within the reference clinical range) were associated with a high risk of VTE. However, elevated values for the Multiplate™ parameters were not associated with a risk of VTE. Our findings suggest that an elevated platelet adhesion function, but not platelet hyperaggregability, play an important role in the pathogenesis of VTE. We believe that our results will provide a firm foundation for additional studies, preferentially prospective, and ultimately find a place in clinical practice.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2018.07.012.

Authorship

MVS and NV contributed equally. They had full access to the database; they performed and interpreted the statistical analyses, written and drafted the manuscript. BC, RM, JMu and DL performed the research and collected the samples. NPM revised the manuscript. MC, JMa, and JF designed the project and collected the data for the Study. JMS analysed and interpreted the data and revised the manuscript, JCS designed the project, collected, analysed and interpreted the data, raised funds for the RETROVE project, as well as editing the manuscript.

Acknowledgements

The authors wish to thank the individuals who participated in the RETROVE Study. Also, we thank Siemens Healthcare Diagnostics, Marburg, Germany for the technical support. We thank Professor Bill Stone for reviewing the manuscript.

This work was supported in part by the Spanish grants: Red Investigación Cardiovascular RD12/0042/0032, FIS PI 12/0612 and FIS PI 15/0269.

Declarations of interest

The authors report no conflicts of interest.

References

[1] M. Nordström, B. Lindblad, D. Bergqvist, T. Kjellström, A prospective study of the incidence of deep-vein thrombosis within a defined urban population, J. Intern. Med. 232 (1992) 155–160.

[2] J.C. Souto, L. Almasy, M. Borrell, F. Blanco-Vaca, J. Mateo, J.M. Soria, I. Coll, R. Felices, W. Stone, J. Fontcuberta, J. Blangero, Genetic susceptibility to thrombosis and its relationship to physiological risk factors: the GAIT study. Genetic analysis of idiopathic thrombophilia, Am. J. Hum. Genet. 67 (2000) 1452–1459, https://doi.org/10.1086/316903.

[3] M.-L. von Brühl, K. Stark, A. Steinhart, S. Chandraratne, I. Konrad, M. Lorenz, A. Khandoga, A. Tirniceriu, R. Coletti, M. Köllnberger, R.A. Byrne, I. Laitinen, A. Walch, A. Brill, S. Pfeiler, D. Manukyan, S. Braun, P. Lange, J. Riegger, J. Ware,

A. Eckart, S. Haidari, M. Rudelius, C. Schulz, K. Echtler, V. Brinkmann, M. Schwaiger, K.T. Preissner, D.D. Wagner, N. Mackman, B. Engelmann, S. Massberg, Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo, J. Exp. Med. 209 (2012) 819–835, https://doi.org/10.1084/jem.20112322.
[4] A.T. Franco, A. Corken, J. Ware, Platelets at the interface of thrombosis, inflammation, and cancer, Blood 126 (2015) 582–588, https://doi.org/10.1182/blood-2014-08-531582.
[5] B. Savage, E. Saldívar, Z.M. Ruggeri, Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor, Cell 84 (1996) 289–297.
[6] J.M. Herbert, A. Bernat, J.P. Maffrand, Importance of platelets in experimental venous thrombosis in the rat, Blood 80 (1992) 2281–2286.
[7] A. Brill, T.A. Fuchs, A.K. Chauhan, J.J. Yang, S.F. De Meyer, M. Köllnberger, T.W. Wakefield, B. Lämmle, S. Massberg, D.D. Wagner, von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models, Blood 117 (2011) 1400–1407, https://doi.org/10.1182/blood-2010-05-287623.
[8] M. Heestermans, S. Salloum-Asfar, D. Salvatori, E.H. Laghmani, B.M. Luken, S.S. Zeerleder, H.M.H. Spronk, S.J. Korporaal, G.T.M. Wagenaar, P.H. Reitsma, B.J.M. van Vlijmen, Role of platelets, neutrophils, and factor XII in spontaneous venous thrombosis in mice, Blood 127 (2016) 2630–2637, https://doi.org/10.1182/blood-2015-10-672766.
[9] S. Montoro-García, M. Schindewolf, S. Stanford, O.H. Larsen, T. Thiele, The role of platelets in venous thromboembolism, Semin. Thromb. Hemost. 42 (2016) 242–251, https://doi.org/10.1055/s-0035-1570079.
[10] S.J. Israels, Laboratory testing for platelet function disorders, Int. J. Lab. Hematol. 37 (Suppl 1) (2015) 18–24, https://doi.org/10.1111/ijlh.12346.
[11] R. Paniccia, R. Priora, A.A. Liotta, R. Abbate, Platelet function tests: a comparative review, Vasc. Health Risk Manag. 11 (2015) 133–148, https://doi.org/10.2147/VHRM.S44469.
[12] B. Jilma, Platelet function analyzer (PFA-100): a tool to quantify congenital or acquired platelet dysfunction, J. Lab. Clin. Med. 138 (2001) 152–163.
[13] E.J. Favaloro, Clinical utility of the PFA-100, Semin. Thromb. Hemost. 34 (2008) 709–733, https://doi.org/10.1055/s-0029-1145254.
[14] R. Paniccia, E. Antonucci, N. Maggini, E. Romano, A.M. Gori, R. Marcucci, D. Prisco, R. Abbate, Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy, Am. J. Clin. Pathol. 131 (2009) 834–842, https://doi.org/10.1309/AJCPTE3K1SGAPOIZ.
[15] L.R. Johnston, P.D. Larsen, A.C. La Flamme, S.A. Harding, Methodological considerations for the assessment of ADP induced platelet aggregation using the Multiplate® analyser, Platelets 24 (2013) 303–307, https://doi.org/10.3109/09537104.2012.694086.
[16] O. Tóth, A. Calatzis, S. Penz, H. Losonczy, W. Siess, W. Siess1, Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood, Thromb. Haemost. 96 (2006) 781–788, https://doi.org/10.1160/TH06-05-0242.
[17] M. Mehrabadi, L.D.C. Casa, C.K. Aidun, D.N. Ku, A predictive model of high shear thrombus growth, Ann. Biomed. Eng. (2016) 1–12, https://doi.org/10.1007/s10439-016-1550-5.
[18] M.K. Puurunen, S.-J. Hwang, C.J. O'Donnell, G. Tofler, A.D. Johnson, Platelet function as a risk factor for venous thromboembolism in the Framingham Heart Study, Thromb. Res. 151 (2017) 57–62, https://doi.org/10.1016/j.thromres.2017.01.010.
[19] C. Hayes, S. Kitahara, O. Tcherniantchouk, Decreased threshold of aggregation to low-dose epinephrine is evidence of platelet hyperaggregability in patients with thrombosis, Hematol. Rep. 6 (2014) 5326, , https://doi.org/10.4081/hr.2014.5326.
[20] C. Martinez, A.T. Cohen, L. Bamber, S. Rietbrock, Epidemiology of first and recurrent venous thromboembolism: a population-based cohort study in patients without active cancer, Thromb. Haemost. 112 (2014) 255–263, https://doi.org/10.1160/TH13-09-0793.
[21] C. Kearon, W. Ageno, S.C. Cannegieter, B. Cosmi, G.-J. Geersing, P.A. Kyrle, P. and D.V. in T.D. Subcommittees on Control of Anticoagulation, Categorization of patients as having provoked or unprovoked venous thromboembolism: guidance from the SSC of ISTH, J. Thromb. Haemost. 14 (2016) 1480–1483, https://doi.org/10.1111/jth.13336.
[22] S.A. Miller, D.D. Dykes, H.F. Polesky, A simple salting out procedure for extracting DNA from human nucleated cells, Nucleic Acids Res. 16 (1988) 1215.
[23] J.C. Souto, L. Almasy, E. Muñiz-Diaz, J.M. Soria, M. Borrell, L. Bayén, J. Mateo, P. Madoz, W. Stone, J. Blangero, J. Fontcuberta, Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2024–2028.
[24] P. Prandoni, Venous and arterial thrombosis: two aspects of the same disease? Eur. J. Intern. Med. 20 (2009) 660–661, https://doi.org/10.1016/j.ejim.2009.02.001.
[25] E. Previtali, P. Bucciarelli, S.M. Passamonti, I. Martinelli, Risk factors for venous and arterial thrombosis, Blood Transfus. 9 (2011) 120–138, https://doi.org/10.2450/2010.0066-10.
[26] J.K. Kruschke, Metric predicted variable with multiple nominal predictors, Doing Bayesian Data Anal, 2n. ed., Academic Press, London, 2015, pp. 583–620.
[27] I. Tirado, J. Mateo, J.M. Soria, A. Oliver, E. Martínez-Sánchez, C. Vallvé, M. Borrell, T. Urrutia, J. Fontcuberta, The ABO blood group genotype and factor VIII levels as independent risk factors for venous thromboembolism, Thromb. Haemost. 93 (2005) 468–474, https://doi.org/10.1160/TH04-04-0251.
[28] V.M. Morelli, M.C.H. De Visser, H.L. Vos, R.M. Bertina, F.R. Rosendaal, ABO blood group genotypes and the risk of venous thrombosis: effect of factor V Leiden, J. Thromb. Haemost. 3 (2005) 183–185, https://doi.org/10.1111/j.1538-7836.2004.01071.x.
[29] M. Vázquez-Santiago, A. Ziyatdinov, N. Pujol-Moix, H. Brunel, A. Morera, J.M. Soria, J.C. Souto, Age and gender effects on 15 platelet phenotypes in a Spanish population, Comput. Biol. Med. 69 (2016) 226–233, https://doi.org/10.1016/j.compbiomed.2015.12.023.
[30] F. Peyvandi, I. Garagiola, L. Baronciani, Role of von Willebrand factor in the haemostasis, Blood Transfus. 9 (Suppl 2) (2011) s3–s8, https://doi.org/10.2450/2011.002S.
[31] P.W. Kamphuisen, J.C. Eikenboom, R.M. Bertina, Elevated factor VIII levels and the risk of thrombosis, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 731–738, https://doi.org/10.1161/01.ATV.21.5.731.
[32] E.J. Favaloro, Utility of the PFA-100 for assessing bleeding disorders and monitoring therapy: a review of analytical variables, benefits and limitations, Haemophilia 7 (2001) 170–179.
[33] M.D. Linden, M.I. Furman, A.L. Frelinger, M.L. Fox, M.R. Barnard, Y. Li, K. Przyklenk, A.D. Michelson, Indices of platelet activation and the stability of coronary artery disease, J. Thromb. Haemost. 5 (2007) 761–765, https://doi.org/10.1111/j.1538-7836.2007.02462.x.
[34] M. Weber, F. Gerdsen, K. Gutensohn, V. Schoder, B. Eifrig, D.K. Hossfeld, Enhanced platelet aggregation with TRAP-6 and collagen in platelet aggregometry in patients with venous thromboembolism, Thromb. Res. 107 (2002) 325–328, https://doi.org/10.1016/S0049-3848(02)00351-1.
[35] E. Tekgunduz, M. Demir, A. Akyol Erikci, S. Akpinar, E. Ozturk, O. Kirkizlar, Sticky platelet syndrome in patients with unexplained venous thrombosis, Turk. J. Hematol. 30 (2011) 48–52, https://doi.org/10.5152/tjh.2011.79.
[36] C. Becattini, G. Agnelli, A. Schenone, S. Eichinger, E. Bucherini, M. Silingardi, M. Bianchi, M. Moia, W. Ageno, M.R. Vandelli, E. Grandone, P. Prandoni, Aspirin for preventing the recurrence of venous thromboembolism, N. Engl. J. Med. 366 (2012) 1959–1967, https://doi.org/10.1056/NEJMoa1114238.
[37] T.A. Brighton, J.W. Eikelboom, K. Mann, R. Mister, A. Gallus, P. Ockelford, H. Gibbs, W. Hague, D. Xavier, R. Diaz, A. Kirby, J. Simes, Low-dose aspirin for preventing recurrent venous thromboembolism, N. Engl. J. Med. 367 (2012) 1979–1987, https://doi.org/10.1056/NEJMoa1210384.
[38] J.I. Weitz, A.W.A. Lensing, M.H. Prins, R. Bauersachs, J. Beyer-Westendorf, H. Bounameaux, T.A. Brighton, A.T. Cohen, B.L. Davidson, H. Decousus, M.C.S. Freitas, G. Holberg, A.K. Kakkar, L. Haskell, B. van Bellen, A.F. Pap, S.D. Berkowitz, P. Verhamme, P.S. Wells, P. Prandoni, EINSTEIN CHOICE Investigators, Rivaroxaban or aspirin for extended treatment of venous thromboembolism, N. Engl. J. Med. 376 (2017) 1211–1222, https://doi.org/10.1056/NEJMoa1700518.

BIBLIOGRAFIA:

1. Fontcuberta J, Borrell M. Hemostasia y trombosis. Conceptos básicos. Manual de técnicas de laboratorio en hematología. 4ta ed [Internet]. © Elsevier; 2014; Cap 22: 665-690.
2. Ren Q., Ye S, Whiteheart SW. The platelet release reaction: just when you thought platelet secretion was simple. Curr Opin Hematol 2008; 106: 2723-2729.
3. Ren Q, Barber HK, Crawford GL, Karim ZA, Zhao C, Choi W, Wanf CC, Hong W; Whiteheart SW. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. Mol Biol Cell 2007; 18: 24-33.
4. Galan AM, Lopez-Vilchez I, Diaz-Ricart M, Navalon F, Gomez E, Gasto C, Escolar G. Serotonergic mechanisms enhance platelet-mediated thrombogenicity. Thromb Haemost 2009; 102:511-519.
5. Duboscq C. Endotelio vascular. HEMATOLOGIA. Volumen 21. Nº extraordinario: 19-20. Fisiología de la hemostasia normal. Agosto 2017.
6. Billaud M, Lohman AW, Johnstone SR, Biwer LA, Mutchler S, Isakson BE. Regulation of Cellular Communication by Signaling Microdomains in the Blood Vessel Wall. Pharmacol Rev 2014; 66:513–569.
7. Von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, Eckart A, Haidari S, Rudelius M, Schulz C, Echtler K, Brinkmann V, Schwaiger M, Preissner KT, Wagner DD, Mackman N, Engelmann B, Massberg S. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo .J Exp Med 2012; 209: 819-835.
8. Preston AE, Barr A. The plasma concentration of factor VIII in the normal population. Br J Haematol 1964;10:238-245
9. Orstavik K, Magnus P, Reisner H, *et al*. Factor VIII and factor IX in a twin population: evidence for a major effect of ABO locus on factor VIII level. Am J Hum Genet 1985; 37:89-101.
10. Gill JC, Endres-Brooks J, Bauer PJ, *et al*. The effect of ABO blood group on the diagnosis of von Willebrand disease. Blood 1987;69:1691-1695.

11. Souto JC, Almasy L, Muñiz-Díaz E, et al. Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. Arterioscler thromb Vasc Biol 2000; 20: 2024-2028.
12. Tirado I, Mateo J, Soria JM, Oliver A, Martínez-Sánchez E, Vallvé C, Borrell M, Urrutia T, Fontcuberta J. The ABO blood group genotype and factor VIII levels as independent risk for venous thromboembolism. Thromb Haemost 2005; 93:468-474.
13. Jenkins PV, O'Donell JS. ABO blood group determines plasma von Willebrand factor levels: a biologic function after all? Transfusion 2006;46:1836-1844.
14. O'Donell J, Boulton FE, Manning RA, *et al*. Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. Arterioscler Thromb Vasc Biol 2002;22:335-341.
15. Shima M, Fujimura Y, Nishiyama T, *et al*. ABO blood group genotype and plasma von Willebrand factor in normal individuals. Vox Sang 1995;68:236-240.
16. Lowe J. The blood group-specific human glycosyltransferases. Baillieres Clin Haematol. 1993;6:465-490.
17. Yamamoto F, McNeil PD, Hakomori S. Human histo-blood group A2 transferase coded by A2 allele, one of the A subtypes, is characterized by a single base deletion in the coding sequence, which results in an additional domain at the carboxyl terminal. Biochem Biophys Res Commum 1992;187:366-374.
18. Yee DL, Sun C.W., Bergeron A.L., Dong J, Bray P.F. Aggregometry detects platelet hyperreactivity in healthy individuals. Blood 2005; 106: 2723-2729.
19. Berger J.S, Becker R.C, Kuhn C, Helms M.J, Ortel T.L, Williams R. Hyperrreactive platelet phenotypes: Relationship to altered serotonin transporter number, transport kinetics and intrinsic response to adrenergic co-stimulation. Thromb Haemost 2013; 109: 85-92.
20. Vázquez-Santiago M, Vilalta N, Cuevas B, Murillo J, Llobet D, Macho R, Pujol-Moix N, Carrasco M, Mateo J, Fontcuberta J, Soria JM, Souto JC. Short closure time values in PFA-100 are related to venous thrombotic risk. Results from the RETROVE study. Thromb Research 2018; 169: 57-63.

21. Kondkar AA, Bray MS, Leal SM, Nagalla S, Liu DJ, Jin Y, Dong JF, Ren Q, Whiteheart SW, Shaw C, Bray PF. VAMP8/endobrevin is overexpressed in hyperreactive human platelets: suggested role for platelet microRNA. J Thromb Haemost 2010; 8:369-378.
22. Yee DL, Bergeron AL, Sun CW, Dong J-F, Bray PF. Platelet hyperreactivity generalizes to multiple forms of stimulation. J Thromb Haemost 2006; 4: 2043-50.
23. Shiffman D, O'Meara E.S, Bare L.A, Rowland C.M, Louie J. Z, Arellano A.R, Lumley T, Rice K, Iakoubova O, Luke M.M, Young B.A, Malloy M.J, Kane J.P, Ellis S.G, Tracy R.P, Devlin J.J, Psaty B.M. Association of gene variants with incident myocardial infarction in the Cardiovascular Health Study. Arterioscler Thromb Vasc Biol 2008; 28: 173-179.
24. Sauer WH, Berlin JA, Kimmel, SE. Selective serotonin reuptake inhibitors and myocardial infarction. Circulation 2001; 104:1894-1898.
25. Fumeron F, Betoulle D, Nicaud V, Evans A, Kee F, Ruidavets J.B, Arveiler D, Luc G, Cambien F. Serotonin Transporter Gene Polymorphism and Myocardial Infarction. Circulation 2002; 105: 2943-2945.
26. Andersson HM, Siegerink B, Luken BM, Crawley JT, Algra A, Lane DA, Rosendaal FR. High VWF, low ADAMTS13, and oral contraceptives increase the risk of ischemic stroke and myocardial infarction in young women. Blood 2012; 119: 1555-60.
27. Matsukawa M, Kaikitia K, Soejima K, Fuchigami S, Nakamura Y, Honda T, Tsujita K, Nagayoshi Y, Kojima S, Shimomura H, Sugiyama S, Fujimoto K, Yoshimura M, Nakagaki T, Ogawa H. Serial changes in von Willebrand factor-cleaving protease (ADAMTS 13) and prognosis after acute myocardial infarction. Am J Cardiol 2007; 100: 758-763.
28. Kaikita K, Soejima K, Matsukawa M, Nakagaki T, Ogawa H. Reduced von Willebrand factor-cleaving protease (ADAMTS 13) activity in acute myocardial infarction. J Thromb Haemost 2006;4: 2490-2493.
29. Crawley JT, Lane DA, Woodward M, Rumley A, Lowe GD. Evidence that high von Willebrand factor and low ADAMTS-13 levels independently increase the risk of a non-fatal attack. J Thromb Haemost 2008; 6: 583-588.
30. Bongers TN, de Bruijne EL, Dippel DW, de Jong AJ, Deckers JW, Poldermans D, de Maat MP, Leebeck FW. Lower levels of ADAMTS13

are associated with cardiovascular disease in young patients. Atherosclerosis 2009; 207: 250-4.
31. Mazetto BM, Orsi FL, Barnabé A, De Paula EV, Flores-Nascimento MC, Annichino-Bizzacchi JM. Increased ADAMTS13 activity in patients with venous thromboembolism. Thromb Res 2012; 130: 889-893.
32. Gandhi C, Ahmad A, Wilson KM, Chauhan AK. ADAMTS13 modulates atherosclerotic plaque progression in mice via VWF-dependent mechanism. J Thromb Haemost 2014; 12: 225-60.
33. Kottke-Marchant K, Importance of platelets and platelet response in acute coronary syndromes. Cleve Clin J Med 2009; 76: S2-S7.
34. Puurunen MK, Hwang S-J, O'Donnell CJ, Tofler G, Johnson AD. Platelet function as a risk for venous thromboembolism in the Framingham Heart Study. Thromb Research 2017; 151: 57-62
35. Hayes C, Kitahara S, Tcherniantchouk O. Decreased threshold of aggregation to low-dose epinephrine is evidence of platelet hyperaggregability in patients with thrombosis. Hematol Rep 2002; 6: 325-328.
36. Weber M, Gerdsen F, Guttensohn K, Schoder V, Eifrig B, Hossfeld. DK Enhanced platelet aggregation with TRAP-6 and collagen in platelet aggregometry in patients with venous thromboembolism. Thromb Res 2002; 107: 325-328 ,
37. Tekgunduz E, Demir M, Akyol Erikci A, Akpinar S, Ozturk E, Kirkizlar O. Sticky platelet syndrome in patients with uninduced venous thrombosis. Turk J Hematol 2011; 30: 48-52.
38. Munz M, Richter GM, Loos BG et al. Genome-wide Association Meta-Analysis of Coronary Artery Disease and Periodontitis reveals a novel shared risk locus. Scientific reports 2018; 12: 8-13678.
39. Campello E, Spieza L, Zabeo E, Maggiolo S, Vettor R, Simioni P. Hypercoagulability detected by whole blood thromboelastometry (ROTEM®) and impedance aggregometry (MULTIPLATE®) in obese patients. Thromb Res, 2015; 135: 548-53.
40. Lundström A, Wallén H, von Arbin M, Jörneskog G, Gigante B, Dembrower KH, Laurencikas E, Laska AC. Clopidogrel resistance after minor ischemic stroke or transient ischemic attack is associated with radiological cerebral small-vessel disease. J Stroke Cerebrovasc Dis, 2015; 24, 2348-57.

41. Lundström A, Laska AC, von Arbin M, Jörneskog G, Wallén H. Glucose intolerance and insulin resistance as predictors of low platelet response to Clopidogrel in patients with minor ischemic stroke or TIA. Platelets, 2014; 25, 102-10.
42. Practical manual: Platelets. Chapter: Laboratory diagnosis of platelet disorders. Catherine P.M. Hayward.
43. Sokol J, Skerenova M, Jedinakova Z, Simurda T, Skonorva I, Stasko J, Kubitz P. Progress in the understanding of Sticky Platelet Syndrome. Semin Thromb Hemost. 2017;43: 008-013.
44. Zhu W, Gregory J, Org E, Buffa JA, Gupta N, Wang Z, Li L, Fu Y, Mehrabian M, Sartor RB, Mcintyre TM, Silverstein RL, Tang WHW, DiDonato JA, Brown JM, Lusis AJ, Hazen SL. Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. Cell 2016; 165: 111-124.
45. Herbert Tilg MD. A gut feeling about thrombosis. N Engl J Med 2018; 374-25.
46. Gorton HJ, Warren ER, Simpson NA, Lyons GR, Columb MO. Thromboelastometry identifies sex-related differences in coagulation. Anesth Analg. 2000; 91:1279-1281
47. Tynngard N, lindahl TL, Ramstrom S, Raf T, Rugarn O, Berlin G. Free oscillation rheometry detects changes in clot properties in pregnancy and thrombocytopenia. Platelets 2008;19: 373-378.
48. Moro L, Reineri S, Piranda D, Pietrapiana D, Lova P, Bertoni A, Graziani A, Defilippi P, Canobbio I, Torti M et al. Nongenomic effects of 17beta-estradiol in human platelets: potentiation of thrombin-induced aggregation through estrogen receptor beta and Src kinase. Blood 2005;105:115-121.
49. Haque SF, Matsubayashi H, Izumi S, Sugi T, Arai T, Kondo A, Makino T. Sex difference in platelet aggregation detected by new aggregometry using light scattering. Endocrine journal 2001; 48: 33-41.
50. Berlin G, Hammar M, Tapper L & Tynngard N. Effects of age, gender and menstrual cycle on platelet function assessed by impedance aggregometry. Platelets 2019;30: 473-479.
51. Pagliari MT, Boscarino M, Cairo A, Manchin I, Rossi F, Martinelli I, Bucciarelli P, Rosendaal FR, Peyvandi F. Evaluation of ADAMTS13 activity as risk factor for deep vein thrombosis. ISTH 2019; Nº OC 15.2. Merlbourne.

52. Anwar M. Al-Awadhi, Seham K. Al-Sharrah, Mehrez M. Jadaon, Faisal Al-Sayegh. Investigating the influence of age, gender and ABO blood group on ADAMTS-13 antigen and activity levels in healthy Arabs. Blood Transfus 2014: 12: 138-40.
53. Vázquez-Santiago M, Vilalta N, Ziyatdinov A, Cuevas B, Macho R, Pujol-Moix N, Carrasco M, Mateo J, Fontcuberta J, Soria JM, Souto JC. Platelet count and plateletcrit are associated with an increased risk of venous thrombosis in females. Results from the RETROVE study. Thromb Res. 2017; 157: 162-164.
54. Bord, S. Vedi, S.R. Beavan, A. Horner, J.E. Compston. Megakaryocyte population in human bone marrow increases with estrogen treatment: A role in bone remodeling? Bone 2000; 27: 397–401.
55. Xin G, Wei Z, Ji C, Zheng H, Gu J, Ma L, Huang W, Morris-Natschke SL, Yeh JL, Zhang R, Qin C, Wen L, Xing Z, Cao Y, Xia Q, Lu Y, Li K, Niu H, Lee KH, Huang W. Metformin Uniquely Prevents Thrombosis by Inhibiting Platelet Activation and mtDNA. Sci Rep 2016; 6:36222.
56. Qin C, Gu J, Jia H, Qian H, Fei X, Li Y, Liu R, Meng W. Platelets activation is associated with elevated plasma mitochondrial DNA during cardiopulmonary bypass. J Cardiothorac Surg 2016; 11: 90
57. Rios DR, Fernandes AP, Figueiredo RC, Guimaraes DA, Ferreira CN, Simoes AC, Carvalho MG, Gomes KB, Dusse LM. Relationship between ABO blood groups and von Willebrand factor, ADAMTS13 and factor VIII in patients undergoing hemodialysis. J Thromb Thrombolysis 2012; 33: 416-21.
58. Bowen DJ. An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS 13. J Thromb Haemost 2003;1:33-40.
59. O'Donnell J, McKinnon TAJ, Crawley JTB, Lane DA, Laffan MA. Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. Blood 2005; 106:6: 1988-1991.
60. McGrath RT, van den Biggelaar M, Byrne B, O'Sullivan JM, Rawley O, O'Kennedy R, Voorberg J, Preston RJ, O'Donnell JS. Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis. Blood 2013; 122: 4107-4110.
61. O'Donell J, Boulton FE, Manning RA, et al. Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. Arterioscler Thromb Vasc Biol 2002;22:335-341

62. Ardillon, L.; Ternisien, C.; Fouassier, M.; Sigaud, M.; Lefrançois, A.; Pacault, M.; Ribeyrol, O.; Fressinaud, E.; Boisseau, P.; Trossaërt, M. Platelet function analyser (PFA-100) results and von Willebrand factor deficiency: A 16-year 'real-world' experience. Haemophilia **2015**, 5, 646–652.
63. Hayward, C.P.; Harrison, P.; Cattaneo, M.; Ortel, T.L.; Rao, A.K. Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. J. Thromb. Haemost. **2006**, 4, 312–319.
64. Nazarian, S.M.; Thompson, J.B.; Gluckman, T.J.; Laws, K.; Jani, J.T.; Kickler, T.S.; Rade, J.J. Clinical and laboratory factors associated with shear-dependent platelet hyper-reactivity in patients on chronic aspirin therapy. Thromb. Res. **2010**, 126, 379–383.
65. Moeller A, Weippert-Kretschmer M, Prinz H, Kretschmer V. Influence of ABO blood groups on primary hemostasis. Transfusion 2001; 41:56-60.
66. Pujol-Moix, N.; Vázquez-Santiago, M.; Morera, A.; Ziyatdinov, A.; Remacha, A.; Nomdedeu, J.F.;Fontcuberta, J.; Soria, J.M.; Souto, J.C. Genetic determinants of platelet large-cell ratio, immature platelet fraction, and other platelet-related phenotypes. Thromb. Res. **2015**, 136, 361–366.
67. Franchini, M.; Lippi, G. Relative risks of thrombosis and bleeding in different ABO blood groups. Semin. Thromb. Hemost. **2016**, 42, 112–117.
68. Zhang, H.; Mooney, C.J.; Reilly, M.P. ABO blood groups and cardiovascular diseases. Review Article. Int. J.Vasc. Med. **2012**, 2012, 641917.
69. Barbalic, M.; Dupuis, J.; Dehghan, A.; Bis, J.C.; Hoogeveen, R.C.; Schnabel, R.B.; Nambi, V.; Bretler, M.;Smith, N.L.; Peters, A.; *et al*. Large-scale genomic studies reveal central role of ABO in sP-selectin and sICAM-1 levels. Hum. Mol. Genet. **2010**, 19, 1863–1872.
70. Chen, Y.; Chen, C.; Ke, X.; Xiong, L.; Shi, Y.; Li, J.; Tan, X.; Ye, S. Analysis of circulating cholesterol levels as a mediator of an association between *ABO* blood group and coronary heart disease. Circ. Cardiovasc. Genet.**2014**, 7, 43–48.
71. Paterson, A.D.; Lopes-Virella, M.F.;Waggott, D.; Boright, A.P.; Hosseini, S.M.; Carter, R.E.; Shen, E.; Mirea, L.;Bharaj, B.; Sun, L.; *et al*. Genome-wide association identifies the *ABO* blood group as a major

locus associated with serum levels of soluble E-selectin. Arterioscler. Thromb. Vasc. Biol. **2009**, 29, 1958–1967.
72. Zhong, M.; Zhang, H.; Reilly, J.P.; Chrisitie, J.D.; Ishihara, M.; Kumagai, T.; Azadi, P.; Reilly, M.P. ABO blood group as a model for platelet glycan modification in arterial thrombosis. Arterioscler Thromb Vasc Biol **2015**, 35, 1570–1578.
73. Zhong, X.; Kriz, R.; Seehra, J.; Kumar, R. N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression. FEBS Lett. **2004**, 562, 111–117.
74. Nagy B , Jin J, Ashby B, Reilly MP, Kunapuli SP. Contribution of the P2Y12 receptor-mediated pathway platelet hyperreactivity in hypercholesterolemia. J. Thromb Haemost 2011; 9: 810-9.